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## **Campylobacter concisus & Inflammatory Bowel Disease**

*– Insights through cultivation and whole-genome sequencing*

Kirk, Karina Frahm

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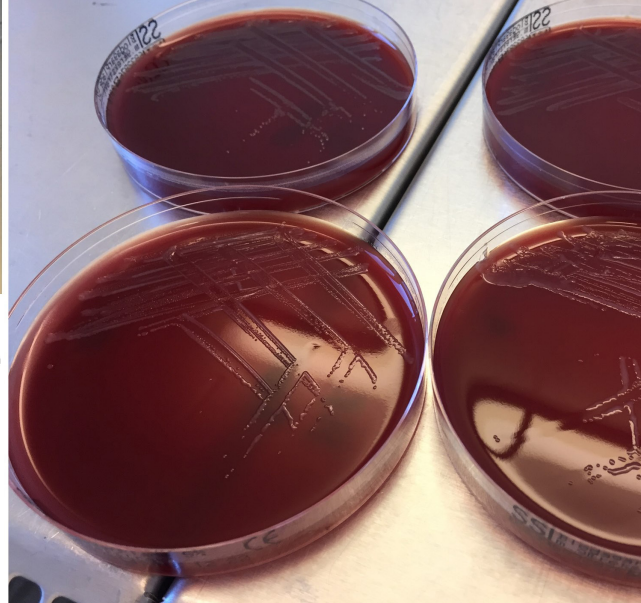
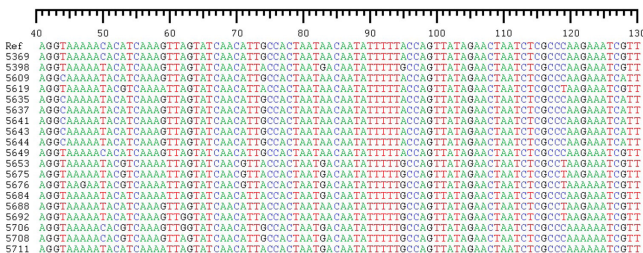
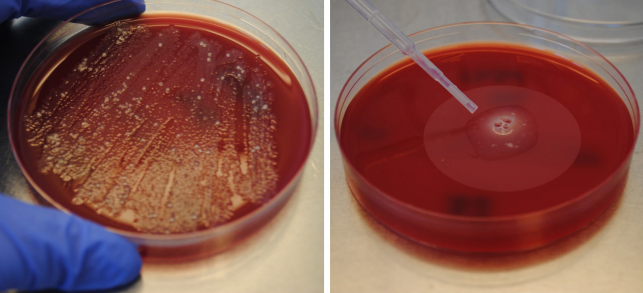
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# CAMPYLOBACTER CONCISUS & INFLAMMATORY BOWEL DISEASE

– INSIGHTS THROUGH CULTIVATION AND WHOLE-GENOME SEQUENCING

BY  
KARINA FRAHM KIRK

DISSERTATION SUBMITTED 2017



AALBORG UNIVERSITY  
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– Insights through cultivation and  
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by

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**AALBORG UNIVERSITY**  
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# ENGLISH SUMMARY

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract, characterized by an excessive immune response to an unknown microbial trigger, in genetically susceptible hosts. The incidence and prevalence of IBD is on the rise on every continent, and currently affects approximately 5 million people worldwide. The current understanding is that dysbiosis of the gut microbiota plays an important part in development and maintenance of epithelial inflammation, and microorganisms with virulent capacities are involved in this process.

*Campylobacter concisus* is a genetically diverse, oral bacterium that has been associated to gastrointestinal diseases such as prolonged diarrhea, Barrett's esophagus and IBD. Previous studies have detected higher amounts of *C. concisus* DNA from IBD patients, but cultivation has been sparse. *In-vitro* studies on the pathogenic properties of this bacterium has shown that it is capable of epithelial cell invasion and disassembly, and can facilitate an increased inflammatory response by inducing cytokine production.

The aim of our studies was to ascertain the prevalence of viable *C. concisus* from the gastrointestinal tract of patients with IBD and healthy controls, and to compare isolates between groups as well as from different sites in the same individual. Over a period of two years, we systematically collected saliva, stool and mucosal biopsies from 57 IBD patients, 28 healthy controls and two patients with persistent diarrhea, to assess the rate of viable *C. concisus*. By implementing a novel cultivation method (the "Aalborg two-step method"), we found that there was a high rate of *C. concisus* from all sample types, in all three groups. However, abundance was highest for the subgroup of IBD patients with the most clinical symptoms, indicating that increased abundance of *C. concisus* may be implicated in disease. Whole-genome sequencing was used for genetic comparison of 104 isolates. By Multi-locus Sequence Typing (MLST) of housekeeping genes, 16S rRNA and 23S rRNA sequences, we found that isolates clustered into two genomospecies, with no correlation to clinical presentation. It was apparent, that individuals were colonized with several different *C. concisus* strains, even in the same anatomic location. We also looked for certain genes associated to pathogenicity and found that these were present in isolates from all groups and from all locations, again with no correlation to clinical presentation.

In conclusion, colonization with *C. concisus* is common and not restricted to the oral cavity or only related to gastrointestinal disease. An explanation could be, that *C. concisus* is a pathobiont – a commensal bacteria with virulent properties that are only expressed under certain conditions, such as dysbiosis of the microbiota in an inflammatory state. Experimental studies are needed to verify that hypothesis.

# DANSK RESUME

Inflammatorisk tarmsygdom (Inflammatory Bowel Disease, IBD) er en kronisk inflammationstilstand i tarmen. Ætiologien er ukendt, men menes at være et uhensigtsmæssigt immunrespons udløst af en eller flere mikroorganismer, hos genetisk prædisponerede individer. Incidencen og prævalensen af IBD er stigende, således er der på verdensplan ca. 5 millioner afficerede mennesker. Det er vist, at en uhensigtsmæssig sammensætning af bakterier i tarmen (dysbiose), spiller en vigtig rolle i udviklingen og vedligeholdelsen af inflammationstilstanden, og at bestemte mikroorganismer med patogene egenskaber kan være medvirkende i denne proces.

*Campylobacter concisus* er en heterogen bakterie, der tidligere har været associeret til langvarig diarré, Barrett's øsophagus og senest, IBD. Tidligere studier har fundet en højere forekomst af *C. concisus* DNA i prøver fra patienter med IBD sammenlignet med raske, men dyrkning har været sparsom. *In-vitro* studier har vist, at *C. concisus* har flere patogene egenskaber, med invasion og ødelæggelse af epitelceller og efterfølgende opregulering af det inflammatoriske respons.

Formålet med vores studier var, at undersøge prævalensen af levedygtige *C. concisus* hos patienter med IBD og raske kontroller og at sammenligne isolater fra de forskellige grupper, samt fra forskellige anatomiske lokalisationer hos den enkelte. I en periode på to år, indsamlede vi spyt og afføringsprøver samt tarmbiopsier fra 57 IBD patienter, 28 raske kontroller og to patienter med persisterende diarré. Vi beskrev en ny metode ("Aalborg two-step method") til dyrkning af biopsier, og fandt at antallet af personer med levedygtige *C. concisus* i tarmen var høj, hos både patienter med IBD og raske kontroller. IBD patienterne med de sværeste symptomer havde en højere forekomst af *C. concisus* sammenlignet med de øvrige grupper, hvilket kunne tyde på, at en større mængde bakterier kunne være associeret til sygdom. Et-hundrede-og-fire isolater blev analyseret ved hel-genom-sekventering og efterfølgende typning af forskellige husholdningsgener, 16S rRNA og 23S rRNA sekvenser. Isolaterne fordelte sig i to genomospecies, der ikke viste association til den kliniske tilstand. Det var tydeligt, at individer kan koloniseres af flere forskellige *C. concisus* stammer, selv i samme lokalisation. Vi undersøgte genomerne for potentielle virulens-gener, tidligere beskrevet for *C. concisus*. Her var der ligeledes ingen forskel mellem grupperne og heller ingen association til klinisk præsentation.

Som konklusion fandt vi, at kolonisering med *C. concisus* er almindeligt, og ikke begrænset til mundhulen eller kun associeret til gastrointestinal sygdom. En forklaring kunne være, at *C. concisus* er en "pathobiont" – en naturligt forekommende bakterie, der kun under de rette omstændigheder kan udvise patogene egenskaber, som kan medvirke til inflammation. Eksperimentelle studier er nødvendige, for at belyse det nærmere.



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Lastly, thank you to my fantastic family – my **parents**, my sister **Sanne**, my husband **Hans** and our children **Mai**, **Søren** and **Mads** – for moral support when needed, for improvised enthusiasm for my research, and for reminding me that not all *Campylobacter* work-related activity is suitable for dinner conversation.

Karina Frahm Kirk, Aalborg 2016

# ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AICC	Adherent and Invasive <i>Campylobacter concisus</i>
AMPs	Antimicrobial proteins
ATCC	American Type Culture Collection
AToCC	Adherent and toxinogenic <i>Campylobacter concisus</i>
BIGSdb	Bacterial Isolate Genome sequences database
BLAST	Basic local alignment search tool
bp	Basepairs
CCUG	Culture Collection, University of Göteborg, Sweden
CD	Crohn's Disease
CFU	Colony Forming Unit
Contigs	Contiguous sequences
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EAC	Esophageal adenocarcinoma
FAP	Familial adenomatous polyposis
GE	Gastroenteritis
GERD	Gastro-esophageal reflux disease
GS	Genomospecies
GWAS	Genome-wide association studies
HC	Healthy controls
Hgb	Hemoglobin
IBD	Inflammatory Bowel Disease
ICD-10	International Classification of disease, version 10
IFN- $\gamma$	Interferon-gamma
IL	Interleukin
IPAA	Ileal pouch-anal anastomosis
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted Laser desorption/ionization Time-of-flight
Mb	Mega bases
MC	Microscopic colitis
mCCDA	Modified charcoal cefoperazone deoxycholate agar
MLST	Multi-locus sequence typing
MS	Mass spectrometry
NOD	Nucleotide-binding oligomerization domain containing protein
NSAID	Non-steroid anti-inflammatory drugs
NTC	Negative template control
PCR	Polymerase Chain Reaction
PFGE	Pulsed field gel electrophoresis
PV	Phase Variation

qPCR	Quantitative Polymerase Chain Reaction
RAPD	Randomly amplified polymorphic DNA
SNPs	Single nucleotide polymorphisms
ST	Sequence Type
TJ	Tight junctions
TLR	Toll-like receptors
TNF- $\alpha$	Tumor necrosis factor alpha
TP	Temperature
UC	Ulcerative colitis
UC-IPAA	Ileal pouch-anal anastomosis due to Ulcerative colitis
WGS	Whole genome sequencing / Next-generation sequencing
Zot	Zonula occludens toxin



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# PREFACE

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*“The real journey of discovery consists not of seeking new landscapes, but in having new eyes.”*

*- Marcel Proust.*

---

Inflammatory bowel disease (IBD) is a chronic, relapsing condition that affects an estimated 2.5 million people in Europe with a steadily rising prevalence all over the world. Repeated periods of medical and surgical treatment in affected individuals can have detrimental effects on their quality of life and ability to work, conjointly being a substantial socioeconomic burden. The etiology of IBD is widely accepted as being an exaggerated immune response in genetically susceptible individuals, and the trigger for this inflammatory overreaction is suggested to be dysbiosis of the intestinal flora, in which pathogenic bacteria can thrive.

*Campylobacter concisus* is an emerging pathogen of the gastrointestinal tract that was originally associated to periodontal disease, and since, premalignant lesions of the esophagus and prolonged diarrhea. While studies have found an association to IBD, coinciding findings in healthy individuals continues to question and contradict a possible correlation. Recent molecular studies on available *C. concisus* isolates have expanded our understanding of the vast genetic diversity that this bacterial species represents, and it is clear that some subtypes harbor pathogenic potentials. However, a sparse number of isolates retrieved from the gut mucosa of IBD patients and healthy individuals have limited the possibility of substantiating this further.

With the expansion of advanced molecular diagnostics such as whole-genome sequencing (WGS) the possibilities for genetic comparison of bacterial isolates have improved tremendously. Over a period of two years, we systematically collected *C. concisus* isolates from saliva, stool and mucosal biopsies of patients with IBD and healthy individuals, in order to perform and optimize cultivation procedures and use whole-genome sequencing methods for isolate comparison. Our hypothesis was that specific pathogenic strains of *C. concisus* are involved in the inflammatory state of gastrointestinal disease.

This project was carried out in collaboration between the Departments of Infectious Diseases, Clinical Microbiology and Gastrointestinal Surgery at Aalborg University Hospital, Denmark, and the Milner Center for Evolution, Department of Biology and Biochemistry at Bath University, United Kingdom.



This thesis is based on the following papers:

1: Karina Frahm Kirk, Hans Linde Nielsen, Ole Thorlacius-Ussing, Henrik Nielsen  
**Optimized cultivation of *Campylobacter concisus* from gut mucosal biopsies in inflammatory bowel disease** Gut Pathogens 2016;1:8-27

2: Karina Frahm Kirk, Hans Linde Nielsen, Guillaume Meric, Ben Pascoe, Samuel K. Sheppard, Ole Thorlacius-Ussing, Henrik Nielsen  
**Comparing *Campylobacter concisus* strains from saliva, faeces and gut mucosal biopsies in inflammatory bowel disease by multi-locus sequence typing using whole genome sequence data.** Draft

3: Karina Frahm Kirk, Hans Linde Nielsen, Guillaume Meric, Ben Pascoe, Samuel K. Sheppard, Ole Thorlacius-Ussing, Henrik Nielsen  
**Virulence-associated genes in *Campylobacter concisus* isolates from patients with inflammatory bowel disease and healthy controls in Denmark.** Draft



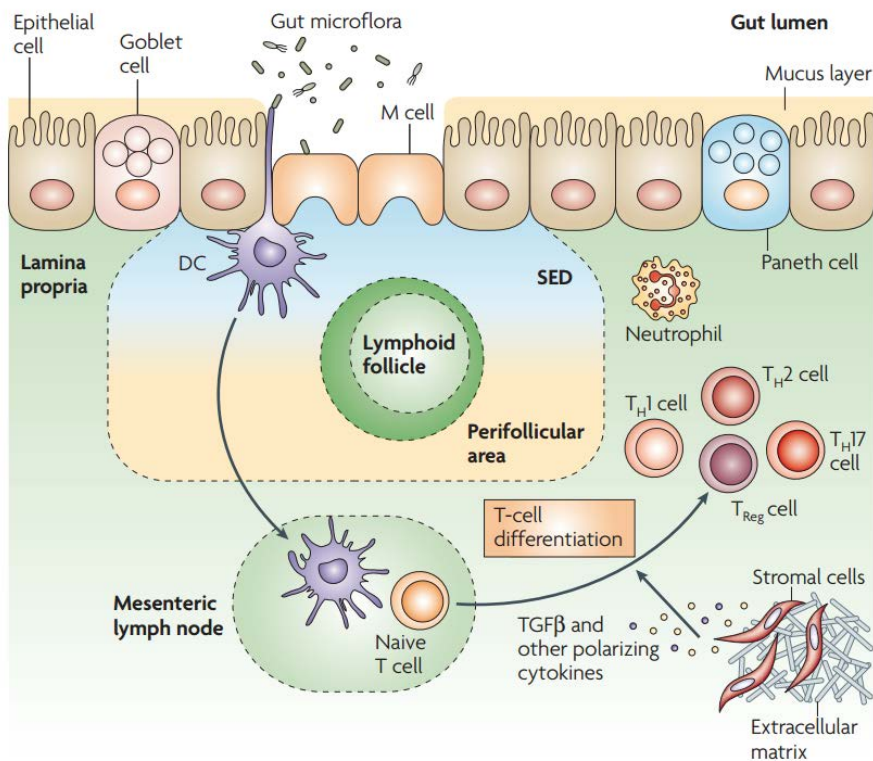
# CHAPTER 1. BACKGROUND

## ***1.1. The gut mucosa and intestinal microbiota***

Intestinal homeostasis depends on complex interactions between commensal gut microbes and epithelial, innate and adaptive immune cells in the gut mucosa. The gut epithelium is the subject of continuous, rapid renewal that occurs without disruption of the functional integrity and permeability.<sup>1</sup> Remarkably, it consists of just one layer of epithelial cells organized into crypts and villi to allow for nutrient absorption as well as water and electrolyte transport. The cells of the epithelium are highly specialized and differentiated to maintain the many physiological functions, and serve as a barrier to pathological intrusions. The most abundant cell type is the absorptive enterocyte, which is adapted for digestive functions. Goblet cells reside throughout the small and large intestine and produce mucus, which is a key element in the barrier function, as well as a source of nutrients for saccharolytic bacteria. Alongside microfold cells, they are also involved in the transport of luminal antigens and bacteria across the epithelial barrier to dendritic cells.<sup>2</sup> In the small intestine, Paneth cells synthesize and secrete antimicrobial proteins (AMPs) such as lysozyme and defensins.<sup>3</sup> Peyer's patches and lymphoid follicles exist imbedded in, and immediately below the epithelial layer in the lamina propria, where macrophages, lymphocytes and other cells of the immune system reside in abundance, for prompt reaction to breach of barrier integrity.<sup>4</sup> Enterocyte cell adhesion is mediated by tight junctions (TJ), adherens junctions and desmosomes; protein structures located between enterocytes. Tight junctions provide a barrier between the gut lumen and the basal membrane, and prevent paracellular passage of fluids, electrolytes and other molecules and are a possible site for functional disruption by bacteria. Cells in the Peyer's patches contribute to maintenance of microbial tolerance, by continuously presenting microbes to cells of the adapted immune system.<sup>1</sup> Dendritic cells present microbial peptides to T-cells, resulting in differentiation into regulatory T cells (Treg) that produce IL-10, a cytokine with anti-inflammatory properties (Figure 1). Neutrophils omit bacterial killing by three mechanisms: phagocytosis, degranulation and later NETosis, a form of self-destruction with expansion of chromatin structures and nucleic proteins with antibacterial properties.<sup>3</sup>

The composition of microorganisms that reside the human gastrointestinal tract is referred to as the intestinal “microbiota” – now recognized as a key player in a magnitude of different illnesses, not only limited to gastrointestinal disease.<sup>5</sup> The volume of bacterial mass increases in the lower intestines, and the distal gut is estimated to harbor up to  $10^{12}$  organisms per gram of luminal content.<sup>1</sup> This complex ecosystem is comprised mostly of bacteria – in the healthy gut dominated by the phyla *Firmicutes* and *Bacteroidetes*, whereas *Proteobacteria* are less dominant under healthy circumstances.<sup>6</sup> The combined genomes of bacterial cells in the intestine is known as the “microbiome”, and impressive worldwide projects have contributed to

our understanding of the intestinal microbiome over time, in both health and disease.<sup>7, 8</sup> The composition of the microbiota is different for every individual and is influenced by both genetic and environmental exposures, resulting in a complex interaction between microbes and host cells throughout life.<sup>9-11</sup> The first colonization of humans occurs during birth and breastfeeding. In the first year of life, the composition of the intestinal microbiota is simple – at about 1-2 years of age, it stabilizes and becomes more diverse, and in preadolescence, it starts to resemble the adult microbiota in diversity, but with distinct microbial communities.<sup>12, 13</sup> In a healthy gut, the microbiome contributes to immune homeostasis, physiological processes and nutrient supply, but perturbations from different factors can alter this homeostasis, resulting in dysbiosis, metabolic disease and inflammatory disorders.<sup>11</sup>



**Figure 1:** The cells of the gut epithelium involved in barrier protection and immune response to pathogenic bacteria. Reprinted by permission from Macmillan Publishers Ltd., from *Cho JH, Nat Rev Immunol. 2008 Jun;8:458-66*

Studies on faecal samples from mono- and dizygotic twins and their family members have shown that the highest degree of similarity is found in samples derived from the same individual, and that phylotype similarity is higher between individuals in the same family as opposed to individuals from different families.<sup>1</sup> Apart from genetic factors, the gut microbiota is influenced by environmental factors, such as exercise

and dietary habits,<sup>14</sup> and even short-term dietary modifications can alter the microbial composition.<sup>15</sup> The gut microbiota may be divided into “mucosa-associated” and “lumen-associated” categories according to anatomical location – the latter may be more unstable, or subject to changes in relation to environmental influences over time.<sup>10</sup> Chiodini and colleagues examined microbial populations from luminal, mucosal and submucosal samples, and found that mucosa-associated populations and submucosal populations are distinct and may have independent associations to intestinal disease.<sup>16</sup>

Studies on the gut microbiota in inflammatory bowel disease have led to a realization that rather than a single organism, the gut microbiota as a whole may be altered, and that this dysbiosis of microbial communities can be explained by interactions between different species in different environmental contexts.<sup>17-20</sup> Pathobionts are organisms that are generally benign, but have the ability to exert pathogenic effects under the right circumstances, which usually involves a disruption of the normal gut-microbe homeostasis. Such organisms could therefore be key players in a prolonged inflammatory response in a dysbiotic environment unless homeostasis is restored.<sup>17</sup>

## 1.2. *Campylobacter*

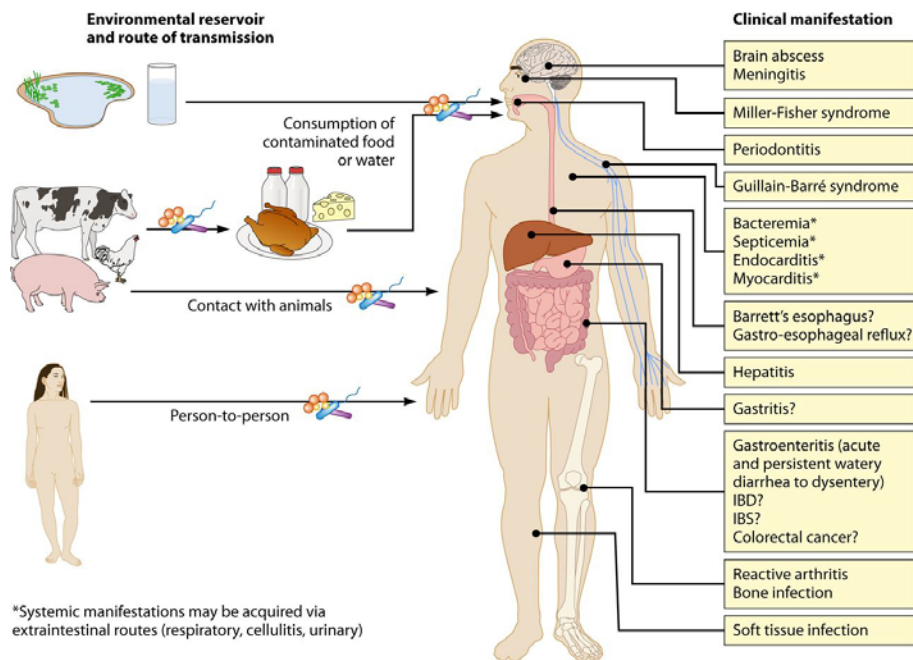
The genus *Campylobacter* belongs to the epsilon class of Proteobacteria in the order of *Campylobacteriales* that includes the *Campylobacteraceae* and *Helicobacteraceae* families. The genera are gram-negative, non-saccharolytic bacteria with microaerobic growth requirements, curved or spiral-shaped and non-spore-forming. They are 0.2 to 0.8  $\mu\text{m}$  wide and 0.5 to 5  $\mu\text{m}$  long and typically motile, with a corkscrew-like movement due to a singular flagellum at one or both ends. Currently, the genus consists of 26 species, two provisional species and nine subspecies.<sup>21</sup> They are diverse in clinical presentations and microbiological features, and at least 17 species have been associated to human disease.<sup>22</sup> Another feature of the *Campylobacter* genus is that they have small genomes (1.6-2.0 Mb) and can establish long-term associations with their host.<sup>23</sup>

The most widely studied species are *Campylobacter jejuni* and *Campylobacter coli*, which are known to cause severe gastroenteritis under the right circumstances and continue to be the leading cause of bacterial gastroenteritis worldwide. Gastroenteritis from *C. jejuni* represents a spectrum from mild to severe disease, but is most often self-limiting. Oral replacement of fluid and electrolytes is the primary therapeutic aim, while hospitalization and antibiotics are seldom required.<sup>24</sup> Reservoirs include poultry, and other animals, as well as water sources in farm environments. Diarrhea due to *Campylobacter* infection (campylobacteriosis) is therefore most commonly associated to consumption of under-cooked poultry that has been contaminated during processing.<sup>25</sup> Post-infectious complications such as recurrent abdominal pain, reactive arthritis and Guillain-Barré syndrome (GBS) have also been described.<sup>26</sup> *Campylobacter jejuni*, *C. coli* and the more uncommon *C. lari* are thermophiles, with an optimum growth temperature of 42 °C, making poultry and birds an ideal reservoir. Emerging *Campylobacter* species, however, have an optimal growth temperature of 37 °C, making humans and other mammals ideal reservoir candidates. As such, *Campylobacter* species are ubiquitous, and sources of *Campylobacter* infections plentiful, as is the spectrum of possible clinical manifestations<sup>21</sup> (Figure 2).

Whole-genome sequencing of *C. jejuni* has revealed extensive genetic variation between isolates. Hypervariable sequences in proteins involved in modification of the capsule, lipooligosaccharide (LOS) and flagellum as well as exchange of DNA between strains, allow the bacterium to continually evolve to adapt to the surrounding environment and evade host immune recognition.<sup>23</sup>

*C. jejuni* has the ability to attach to human intestinal cells, subsequent to adherence through proteins on the bacterial surface that interact with the epithelial cells. This induces a proinflammatory response, characterized by IL-8 production that in turn recruits inflammatory cells such as neutrophils, dendritic cells and macrophages to enhance the inflammatory response resulting in diarrhea and bacterial clearance. It has been shown, that strains of *C. jejuni* have the ability to invade intestinal cells, but the role of this function in the pathogenesis of *C. jejuni* infection has not been

established. While cells of the innate immune system dominate the response to *C. jejuni* infection, human sera antibodies to bacterial components such as flagella and LOS have also been determined.<sup>23</sup>



**Figure 2:** Sources of *Campylobacter* infection and the clinical manifestations. From Kaakoush et al. *Clin. Microbiol. Rev.* 2015;28:687-720. Reprinted with permission from ASM Press.

Recognition of the importance of non-*jejuni*/non-*coli* *Campylobacter* species has increased, especially in the past two decades. These organisms are slow-growing and susceptibility to different antibiotics in selective medias limit their routinely detection in diagnostic laboratory settings. Reservoirs and disease associations of these emerging *Campylobacter*s are still vaguely described, but the pathogenic potential of several of these species are becoming of increasing interest. This thesis focuses on *C. concisus*, whereas detailed descriptions of clinical and microbiological features of other emerging *Campylobacter* species can be found elsewhere.<sup>27</sup>

### **1.3. *Campylobacter concisus***

Tanner et al. first described *Campylobacter concisus* in relation to humans in 1981, where it was isolated from the oral cavity of patients with periodontal lesions, and proposed to contribute to gingival destruction.<sup>28</sup> It is a slow-growing, fastidious organism, with an optimal growth temperature of 37 °C, and a single polar flagellum. Biochemically, the species is urease and catalase negative, and oxidase positive.<sup>29</sup> *C. concisus* is usually susceptible to ciprofloxacin and macrolides, but resistance to ciprofloxacin has been reported.<sup>30</sup> While an animal reservoir has not been identified, it has been isolated from domestic pets,<sup>31, 32</sup> fresh meat and poultry.<sup>33</sup> Since the first isolation from the oral cavity, different studies have found that *C. concisus* was not only associated to disease but could be isolated and detected from healthy individuals as well.<sup>34-36</sup> There has only been reported one case of extra-oro-intestinal infection with *C. concisus*, in a polymicrobial brain abscess from a male with intracranial carcinoma,<sup>37</sup> as well as one case of bacteremia with *C. concisus* following gastroenteritis, also in a male with a history of carcinoma.<sup>38</sup>

#### **1.3.1. Association to human gastrointestinal disease**

The first reports of a possible association to gastrointestinal disease were from children with diarrhea, initially reported from Sweden.<sup>39</sup> A large study conducted at the Red Cross Children's Hospital in South Africa over a period of almost two decades isolated *Campylobacter* species in 6.006 cases, and found that *C. concisus* accounted for 25.02% of these, thereby being the second most prevalent species after *C. jejuni* (32.57%).<sup>40, 41</sup> Several studies conducted in Denmark have also found a high prevalence of *C. concisus* in diarrheic stool samples from both adults and children,<sup>42</sup> as well as immunocompromised individuals.<sup>43</sup> Engberg et al. isolated *C. concisus* from the stools in a minority of healthy individuals, and a study from Belgium found that *C. concisus* was present in the stools of healthy children.<sup>44</sup> In a large study on 8.302 patients with diarrhea in Denmark, the incidence of *C. concisus* was found to be almost as high as for *C. jejuni*. From 11.314 faecal samples, 441 *C. concisus* isolates were recovered from 400 patients. *C. jejuni/coli* was isolated in 541 cases from 489 patients. The study included 108 healthy volunteers, of which none had a *C. concisus* culture positive stool sample.<sup>30</sup> Nielsen et al. described the clinical manifestations of enteric *C. concisus* infection, which include prolonged diarrhea with a milder course compared to *C. jejuni/coli*, also applicable for the pediatric population.<sup>45</sup> Patients also had lower levels of serum inflammatory markers and a lower prevalence of fever, chills, mucus and blood in their stools, and interestingly, a possible association to development of microscopic colitis (MC) was noted.<sup>46</sup> Recently, Underwood and colleagues used qPCR for *C. concisus* detection in faecal samples from patients with diarrhea, using cut-off values validated for *C. jejuni* to assess clinical relevance levels. The prevalence of clinically relevant levels of *C. concisus* in samples was 18%, compared to only 5% for *C. jejuni/coli*.<sup>47</sup> However, these results were not correlated



to cultivation rates, which would be interesting in terms of establishing clinical cut-off values.

Barrett's esophagus (BE) is a premalignant condition related to chronic gastroesophageal reflux disease (GERD). A study from 2007 found *C. concisus* in biopsies from four out of seven patients with this condition but in none of the seven controls.<sup>48</sup> These findings were confirmed in a later study that sampled biopsies from patients with BE, GERD and esophageal adenocarcinoma (EAC), as well as healthy controls. In this study, *C. concisus* was the predominant species in BE and GERD, but not in healthy controls or EAC. Interestingly, the authors found a strong association between *C. concisus* colonization and epithelial IL-18 production, a pro-inflammatory cytokine also involved in the pathogenesis of gastric cancer.<sup>49</sup>

The first indication for a possible association between *C. concisus* and IBD was reported when *Campylobacter* species in biopsy specimens from children with CD were analysed, revealing a significantly greater presence of *C. concisus* DNA compared to biopsies from healthy controls.<sup>50, 51</sup> Similar results were later described by Man and colleagues, who observed that 65% of faecal samples from patients with CD were positive for *C. concisus*, compared to 33% of faecal samples from healthy controls.<sup>51</sup> The same findings have followed for patients with UC. Mahendran and colleagues found that biopsies from both CD and UC patients analyzed by PCR had a higher prevalence of *C. concisus* compared to healthy controls ( $p < 0.05$ ).<sup>52</sup> In that study, the authors also compared the prevalence in four anatomical sites and found that *C. concisus* preferentially colonized the proximal large intestine in IBD patients, although it was present in all parts examined.<sup>52</sup> Mukhopadhyaya and colleagues in Scotland also detected *C. concisus* more often in biopsy specimens from adults with UC compared to controls ( $p = 0.0019$ ).<sup>53</sup> In contrast, the same research group examined the microbiota of children with newly diagnosed CD and found that, although *Campylobacter* species in general were common in pediatric biopsies, there was no difference in detection of *C. concisus* when comparing IBD patients and controls.<sup>54</sup> Zhang et al. found that there was no difference in the prevalence of *C. concisus* in saliva samples from IBD patients and healthy controls, and that *C. concisus* DNA was prevalent in the majority of individuals tested (18/18 IBD patients and 57/59 HC).<sup>36</sup> These results indicate that the oral cavity is a possible reservoir for *C. concisus*, and that an association to IBD is only related to isolates present in the gut.

### 1.3.2. Species diversity - Isolation and identification methodology

The preferred media for isolation of *Campylobacter jejuni*/*Campylobacter coli* in clinical microbiology laboratories is the modified charcoal cefoperazone deoxycholate agar (mCCDA).<sup>55</sup> This method is simple and reliable for detection of *C. jejuni*, but *Campylobacter* species that are susceptible to cefoperazone in this media cannot be detected. While there is no gold standard for isolation of emerging *Campylobacter* spp. such as *C. concisus*, the best-described methodology is isolation

by filtration onto non-selective media, by the so-called 'Cape Town protocol'.<sup>56</sup> Using polyagarose filters with a pore size of 0.4-0.8  $\mu\text{m}$  the spiraling movements of *Campylobacter* spp. can be utilized to separate these from other enteric bacteria in faecal samples.<sup>57</sup>

Most *Campylobacter* species metabolize amino acids or tricarboxylic acid cycle intermediates for growth in microaerobic conditions. Six species, *C. concisus*, *C. curvus*, *C. gracilis*, *C. rectus*, *C. showae*, and *C. ureolyticus* have been described to require hydrogen or formate as an electron donor in this process.<sup>27</sup> Recently, Lee et al. examined the growth of *C. concisus* in different atmospheres and observed that oral and enteric *C. concisus* isolates grew in anaerobic conditions without the presence of  $\text{H}_2$ , formate or fumarate, although the presence of  $\text{H}_2$  increased growth.<sup>58</sup> Different methods for attaining microaerobic and anaerobic atmospheres for cultivation of *Campylobacter* species are available; most commonly the gas generating sachets or automated gas delivery systems. In previous studies, no significant difference in cultivation rates between the two systems has been observed, although it appears that colony size may increase when using the automated gas delivery system.<sup>59-61</sup>

Traditional laboratory methods to identify *Campylobacter* species have included various phenotypic, biochemical and serological tests, but as the number of *Campylobacter* species is on the rise, traditional tests become less useful in differentiating isolates from one another.<sup>62</sup> Since the isolation of *C. concisus* from different clinical samples from patients with enteric disease and healthy controls, different typing systems have been used to characterize the species by genetic profiling. Vandamme et al. proposed a genetic diversity of *C. concisus* already in 1989, where DNA-DNA hybridization was used to compare *C. concisus* strains from diarrheic patients with oral reference strains, revealing only 42-50% matched values.<sup>63</sup> These findings were supported in a later study from Belgium, where *C. concisus* strains from 37 children with diarrhea showed that 94.6% of these had distinct randomly amplified polymorphic DNA (RAPD) profiles.<sup>44</sup> In 2002, Matsheka et al. described a typing protocol using pulsed field gel electrophoresis (PFGE), where 51 of 53 strains had distinct PFGE profiles,<sup>64</sup> findings later supported by RAPD analysis.<sup>65</sup> A Danish study from 2002 used a lectin typing system based on the bindings of lectins to LPS extracts of *C. concisus*, from stools samples from patients with a variety of clinical conditions, including HIV, malignancies and IBD. The system grouped 44 strains into 13 patterns that did not correlate with the different clinical diseases.<sup>66</sup> In 2005, Engberg et al. found that SDS-PAGE protein profiling and PCR amplification of the 23S rRNA gene resulted in clustering of 39 *C. concisus* isolates into two genomospecies (GS), while RAPD profiling found 34 isolates to have distinct profiles.<sup>67</sup> Several other research groups have also proposed a similar grouping of *C. concisus* isolates into two major genomospecies. Aabenhus et al. investigated 62 *C. concisus* isolates of mixed enteric and oral origin by AFLP profiling and found a clustering into four groups that were divided in two GS. Isolates from immunodeficient patients were included in this study, and interestingly, these isolates

were all assigned to AFLP cluster 4, from which the authors' proposed that the GS may reflect variations in the pathogenic potential of isolates.<sup>68</sup> Recently, Nielsen et al. conducted an MLST analysis of 63 faecal and four oral *C. concisus* isolates from 49 patients with different clinical illnesses (29 with diarrhea, eight with bloody diarrhea, seven with MC, 5 with CD). Similar to previous findings, the isolates clustered into two major groups, but subgrouping was independent of clinical presentation.<sup>69</sup> Today, identification of *Campylobacter* species is more readily available by rapid molecular methods in a laboratory setting. Polymerase chain reaction (PCR) methods can amplify specific regions of DNA that are specific to different species. For *C. concisus*, the most widely used primers target the 16S rDNA, 23S rDNA or chaperonin-60 (cpn60) genes.<sup>31, 50, 70</sup> Introduction of MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) has enabled rapid typing of isolates, verified to be able to distinguish *C. concisus* from other *Campylobacter* species, and different *C. concisus* isolates from each other with unique spectras.<sup>71</sup> With expansion of the *C. concisus* reference library, MALDI-TOF MS could be useful in strain differentiation in a routine laboratory.

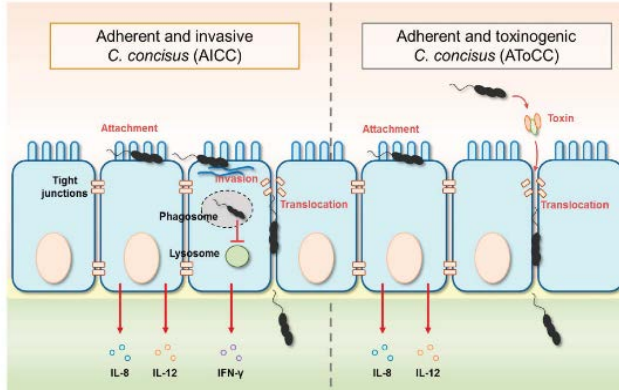
The most comprehensive method for genetic typing is whole genome sequencing, which will be discussed in detail later. Presently, nine *C. concisus* genomes have been sequenced and are available at GenBank in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). These include three strains from CD patients (UNSWCD, UNSW2 and UNSW3), five from diarrheal faecal samples (13826/BAA-1457, UNSW1, UNSWCS, ATCC 51562), one from a faecal sample from a healthy individual (ATCC 51561) and one from the saliva of a patient with periodontitis (ATCC 33237). Only one isolate (13826 also known as BAA-1457) has been fully sequenced with complete closing of the genome. Kaakoush et al. were the first to compare the genomes of *C. concisus*, when they analysed the 13826 reference strain and the UNSWCD strain in 2011.<sup>72</sup> They found that these two genomes only had 76% genes homology, and had significant differences in flagellin glycosylation pathways.<sup>72</sup> Deshpande and colleagues subsequently conducted a comprehensive comparative study of eight of the above mentioned genomes and found that *C. concisus* strains could be grouped by pathogenic factors such as adherence, invasiveness and motility.<sup>73</sup> Very recently, Chung et al. sequenced 27 oral *C. concisus* strains from IBD patients and HC and described two genomic islands (CON\_PiiA and CON\_piiB) that contained proteins homologous to a type IV secretion system, and effector proteins possibly involved in intracellular survival.<sup>74</sup> The authors found that CON\_PiiA islands were found in 3/8 enteric strains and none of the oral strains, and may therefore be associated to virulence (These genomes are at the time of writing not publically available).

### 1.3.3. Pathogenic potential

As is the case for most pathogenic bacteria, virulence mechanisms can be classified by effect – invasion of host cells, evasion of host immune responses and induction of cell damage or dysfunction.<sup>75</sup> Studies from the gastric microbiota have shown that *C. concisus* can tolerate wide variations in pH,<sup>76</sup> and remains transcriptionally active in acidic environments.<sup>77</sup> *C. concisus* has a single polar flagellum that allows for penetration through mucus, enabling contact with the gut epithelial cells, through flagellum-microvilli interaction.<sup>78</sup> In-vitro studies have shown that *C. concisus* can invade host cells<sup>79</sup> and that this potential is increased in the presence of the inflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$ .<sup>78</sup> Other in-vitro studies have shown that *C. concisus* isolates are capable of producing biofilm,<sup>80</sup> and of inducing apoptotic leaks in epithelial cells.<sup>79</sup> Interestingly, both of these properties were present in both faecal and oral isolates. In 2011, Kaakoush et al. found that strains isolated from chronic intestinal diseases were 500-fold more invasive than isolates from acute gastroenteritis cases and healthy controls. A plasmid containing various virulence determinants was detected in the chronic intestinal strains, which could explain the exceptional invasive potential of such isolates.<sup>72</sup> The putative virulence factor from this plasmid is exotoxin9/DnaI that is associated to increased survival in epithelial cells. The same research group showed that exotoxin9/DnaI levels were significantly higher in faecal samples from CD patients compared to healthy controls.<sup>81</sup> Kaakoush et al. also described in detail the secretome of *C. concisus* in which an abundance of secreted proteins known to be associated to virulence in other species, were identified.<sup>82</sup> The same group also described upregulations of inflammatory pathways in the innate immune response by transcriptomic and proteomic analysis of infected macrophages.<sup>83</sup> One of the key elements involved in barrier damage is believed to be Zot (Zonula occludens toxin), a toxin that targets intercellular tight junctions. The *zot* gene was initially described in *Vibrio cholera*, where it encodes a protein capable of disassembling tight junctions between epithelial cells leading to increased permeability, an important link to the clinical finding of secretory diarrhea.<sup>84</sup> Deshpande et al. investigated the epithelial response to Zot in Caco-2 cells using RNAseq and found that *PAR2* expression, cytoskeletal remodeling and cytokine production was upregulated following Zot exposure.<sup>85</sup> The changes in tight junctions after exposure to Zot are reversible and by investigation of *zot* sequences from different isolates, Mahendran et al. described that polymorphisms in the *zot* gene may be associated to clinical disease, rather than just the presence of *zot* by itself.<sup>86</sup> Recently, the same research group investigated several other *Campylobacter* species for the presence of *zot*, and identified twelve *zot* genes from nine species/subspecies that could be divided into two main groups, related to the diversity of the encoded proteins.<sup>87</sup>

Based on some of the virulence properties mentioned above, Kaakoush et al. have proposed a division of pathogenic *C. concisus* isolates into two groups: adherent and invasive *C. concisus* (AICC), and adherent and toxinogenic *C. concisus* (AToCC),

similar to classification systems used for *E. coli* of which some subtypes have previously been associated to CD.<sup>29, 88</sup> (figure 3).

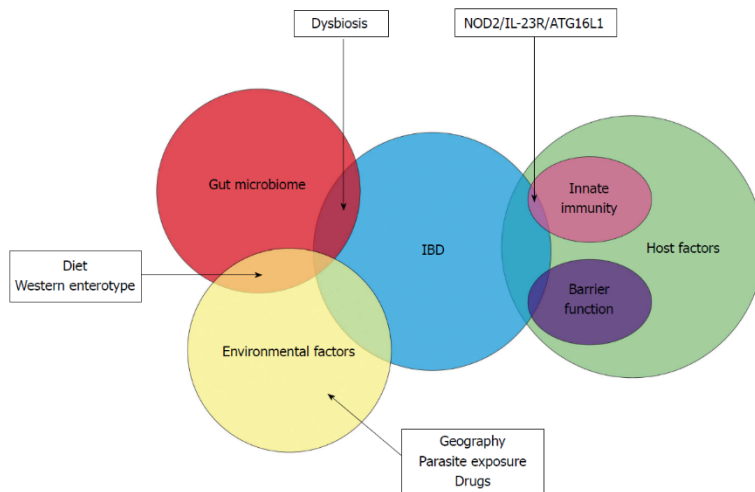


**Figure 3:** Pathogenic *C. concisus* strains can be divided into two types (AICC) and (AToCC) From: Kaakoush et al. *Inflamm Bowel Dis* 2014;20:2189-2197 Reprinted with permission from Wolters Kluwer Health, Inc.

#### 1.4. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is comprised of two major diseases: Crohn's disease (CD) and Ulcerative colitis (UC) (ICD-10: K50-K51) – both characterized by an excessive immune response to an unknown microbial trigger, in genetically susceptible hosts<sup>89, 90</sup> (Figure 4). Microscopic colitis (MC) is another chronic illness in the IBD group, but the etiology and clinical course of the disease distinguishes it from UC and CD, and it will not be discussed in more detail in this thesis. The etiology of UC and CD are believed to be same or closely related, although an exact pathogenic mechanism has yet to be described. Genetic studies have identified susceptible loci with an association to IBD, and a recent genotype association study proposed that disease location is in part genetically determined, while disease presentation and behavior are not.<sup>91</sup> The incidence and prevalence of CD and UC are on the rise on every continent, and currently affects approximately one in 200 people in Europe and 5 million people worldwide.<sup>92</sup> The direct expenditures related to health-care are estimated to be €4.6-5.6 billion annually in Europe and indirect costs such as reduced work capacity are thought to exceed that number. Moreover, reduced quality of life, and social stigmata can influence the patients and their families for long periods of time.<sup>93</sup> Historically, the incidence of IBD has risen steadily in Western industrialized countries since the recognition of the disease in the 1900's. Furthermore, newly industrialized countries in Asia and the Middle East with dense populations continue to describe an increase in IBD incidence, indicating that the burden of disease is surging worldwide.<sup>94</sup>

Many studies have examined genes and genetic loci that could be implicated in the development of IBD and as of 2016, 163 susceptibility loci have been identified. Some loci are associated to either UC or CD exclusively, while others are shared in both diseases. The implicated genes are related to different aspects of intestinal homeostasis, such as barrier function, epithelial restitution and autophagy.<sup>89</sup> One of the most widely studied genes is *NOD2*, which was the first to be associated to CD. *NOD2* is a protein with a key function in immunoregulation, expressed in gut epithelial cells. Under normal circumstances, it detects peptidoglycans from microbes in the gut lumen, and activates the production of mucin and antimicrobial peptide through complex signaling processes. Loss-of-function mutations in the *NOD2* gene are associated to the development of CD, by loss of protective inflammatory response and barrier function, which can lead to dysbiosis and chronic inflammation.<sup>95</sup> Other genes that have been associated to IBD through an altered immune response to bacteria are *IL23R* and *ATG16L1*. With the expansion of genome-wide association (GWAS) studies, it has become apparent that genetic risk is associated to disease sub phenotypes<sup>91</sup> and that the interaction between genetic determinants and dysbiosis is a crucial element in IBD development.<sup>96</sup>



**Figure 4:** A Venn-diagram depicting the overlapping etiologic factors in IBD. From *Hold et al. World J Gastroenterol. 2014;20:1192–1210* Reprinted under the Creative Commons Attributions Licence (CC- BY).

The increase in IBD incidence in developing countries as these become industrialized underlines the fact, that environmental factors also play a part in IBD pathogenesis and over the years, different exposures have been examined. The first associative factor to be described was smoking – which increases the risk of developing CD while having a protective effect on the development, maintenance and severity of UC.<sup>97</sup> Other environmental factors that have been examined are use of NSAIDs, oral

contraceptives, psychiatric disorders and diet.<sup>98</sup> The use of antibiotics during childhood has also been shown to be associated to the development of IBD,<sup>99</sup> although a direct causal effect is difficult to establish as symptoms of IBD may have been present years prior to diagnosis leading to subsequent antibiotic treatment.

Various specific enteric pathogenic bacteria have been proposed to be involved in the pathogenesis or exacerbation of IBD. Dating back as far as 1913, Dalziel, a Scottish surgeon, first described CD by comparing it to Johne's disease in cattle which is caused by *Mycobacterium avium* subspecies *paratuberculosis*.<sup>100</sup> Lack of epidemiological support and unresponsiveness to traditional antimycobacterial drugs have contradicted this association, but the theory is still disputed.<sup>101</sup> Another bacterium of interest has been pathogenic *E. coli* strains, after descriptions of certain adherent strains being present in mucosal tissue of patients with CD, but not in healthy controls.<sup>88, 102, 103</sup> Similarly, alpha-haemolysin producing *E. coli* have been shown to be capable of producing focal leaks in colonic epithelia.<sup>104</sup> In a Danish population-based cohort study the authors found an increased risk of IBD diagnosis following *Campylobacter* and *Salmonella* gastroenteritis (RR=5.4-9.8 in the first year after the positive faecal sample).<sup>105</sup> However, there is also an increased risk of IBD diagnosis after having a negative stool test, indicating that patients who develop IBD have a history of increased stool sampling, probably parallel to disease evolution.<sup>106</sup> *Clostridium difficile*, known to cause antibiotic-associated diarrhea and pseudomembranous colitis, has also been investigated in relation to IBD patients, as studies have shown that up to 10% of IBD patients will develop *C. difficile* infection at some point.<sup>107</sup> However, there is little literature to support a causative association and the existence of *C. difficile* in IBD may merely be due to an inflammatory status, although positive findings in IBD should lead to eradication treatment.<sup>108</sup> In contrast, studies have proposed an inverse relationship between IBD and *Helicobacter pylori*, based on *in-vitro* studies have shown that *H. pylori* infected patients have lower systemic levels of the proinflammatory cytokines type 1 IFN-gamma and IL-12,<sup>109</sup> recently confirmed in a large meta-analysis.<sup>110</sup> Likewise, other bacterial genera have been suggested to offer protective effects in IBD, for example *Bacteroides*, *Bifidobacterium*, *Lactobacillus* and *Faecalibacterium*, by several mechanisms including down-regulating production of inflammatory cytokines.<sup>11</sup> *Faecalibacterium prausnitzii* ferments dietary fibers and produces butyrate, a short-chain fatty acid, which is a primary energy source for colonic epithelial cells. Several studies have shown that the prevalence of *F. prausnitzii* is depleted in both UC and CD patients, and it has been proposed that probiotic treatment with *F. prausnitzii* strains could be beneficial in IBD management.<sup>111-113</sup>

Specific viruses have also been examined for possible associations to IBD, including Varicella zoster, Cytomegalovirus and Epstein-Barr virus. The implications of these virus in the pathogenesis of IBD is uncertain and comprehensive studies on this topic are lacking.<sup>114</sup> An interesting study by Norman and colleagues focused on characterizing the human virome, the viral counterpart to the bacterial genome. They

found, that while IBD patients have decreased bacterial diversity in faecal samples, the viral diversity was increased – especially in the form of bacteriophages. Bacteriophages are responsible for the horizontal transfer of genetic material among bacterial communities, including those for pathogenesis, and the inverse relationship between bacterial and viral diversity in IBD may provide interesting information about the conformation of bacteria involved in the inflammatory process.<sup>115</sup>

The microbiota of IBD patients has been reported to express less diversity than in healthy individuals, characterized by fewer *Firmicutes* and *Bacteroidetes* that generally express anti-inflammatory properties and more *Proteobacteria* with proinflammatory properties.<sup>116</sup> Genome-wide associations studies (GWAS) indicate that IBD is biologically heterogeneous and metagenomics analysis indicate that this is also the case for the microbiota.<sup>117</sup> Shifts in microbial composition may rely on severity of inflammation, disease states and genetic disposition, as well as environmental modulations such as anti-inflammatory or antibiotic drug use. On a cellular level, bacterial interaction with gut mucosa cells may rely on the mucosa composition, barrier defects, and the local host-mediated inflammatory response. Recognizing the diversity of IBD manifestations in different individuals is essential in attempting to improve treatment modalities and patient care, and individualized treatments are becoming of increasing interest.<sup>118</sup>

While clinical symptoms overlap, pathological findings separate and characterize the two disorders distinctly (Table 1), discussed in detail below.

**Table 1:** Differential diagnosis of UC and CD.

<b>Findings</b>		<b>UC</b>	<b>CD</b>
<b>Clinical</b>	Perianal disease	Rare	Common
	Fistulas		
	Abscesses		
	Strictures		
<b>Endoscopic</b>	Rectal involvement	Always	Unusual
	Pattern	Continuous	Skip lesions
<b>Imaging</b>	Ileal involvement	Rare (Backwash)	75%
<b>Histological</b>	Inflammation depth	Mucosa/submucosa	Transmural
	Granulomas	No	20%
	Fissures	Rarely	Common
	Crypt architecture	Distorted	Normal
	Crypt abscesses	Yes	Yes

Adapted from Baumgart, D. C. & Sandborn, W. J. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* **369**, 1641-1657 (2007).



### **1.4.1. Crohn's Disease**

Crohn's disease is defined by inflammation of all layers in the intestinal wall and skip lesions that can affect the entire gastrointestinal tract, from the mouth to the anus. The most common symptoms at the time of diagnosis include diarrhea, abdominal pain, fever, and stools with blood or mucus or both. Complications associated with Crohn's disease are related to the transmural lesions, with risks of perforations, fistulas and strictures. Diagnosis is based on a combination of clinical findings, endoscopic features such as cobble stoning, fissures, fistulas and ulcers, as well as histological features such as inflammatory cell infiltration, crypt irregularity or granulomas.<sup>119</sup> Imaging studies, such as CT or MRI can assist in determining the extent of disease and possible complications such as sclerosing cholangitis. The Montreal classification can be used to describe the extent of disease, and disease severity, encumbering age at diagnosis, extent of inflammation and disease behaviour.<sup>120</sup> The disease is slightly more common in women (1.3-1.5:1), and age at disease onset is approximately 15-30 years. It has been reported, that 13-20% of CD patients have a chronic active course of disease activity, 67-73% have a chronic intermittent course and 10-13% are in sustained remission.<sup>119</sup>

### **1.4.2. Ulcerative colitis**

The inflammation in UC is limited to the mucosa and submucosal layers of the intestinal wall. The rectum is always affected, wherefrom inflammation spreads proximally in a continuous fashion. Apart from cases with backwash ileitis, the ileum is never involved, and the severity of symptoms and degree of colonic involvement is used to classify the disease.<sup>120</sup> Complications are less common than in CD, and are abscesses and necrosis of colonic tissue, that can ultimately result in toxic megacolon with perforation. Ulcerative colitis affects men and women almost equally, and the age of onset has a bimodal peak between the ages of 15-30 and 50-70.<sup>121</sup> The main symptoms at presentation are bloody diarrhea, pus or mucus in the stool, and abdominal cramping.<sup>122</sup> Endoscopic findings include loss of vascular pattern, granularity, friability with spontaneous bleeding, erosions and ulcerations. Pathological findings from microscopic examinations of mucosal biopsies are distortion of crypt architecture, immune cell infiltration of the lamina propria, mucin depletion erosion or ulcerations.<sup>121</sup> A combination of clinical, endoscopic and histological findings comprise the diagnosis of UC, though infectious and other non-infectious causes of the symptoms must also primarily be investigated and ruled out. It has been reported that the distribution of disease activity is relatively constant, with 50% in clinical remission at any given time, while 20-30% will require surgery at some point, and 24% will have undergone a colectomy 10 years after diagnosis.<sup>123</sup> Medical treatment regimens include systemic or rectal application of 5-aminosalicylic acid (mesalazine) and/or corticosteroids, according to individual response and clinical presentation. Treatment of severe UC can be attempted with more complex immunological drugs such as tacrolimus, ciclosporin or infliximab, that specifically

target proinflammatory cytokines. Individual treatment regimens depend on disease extent and severity, and detailed treatment algorithms are available.<sup>121</sup> Emergency surgery is performed in case of severe complications such as toxic megacolon or perforation, and can also be performed electively in case of intolerance or non-responsiveness to medical treatment.<sup>121</sup>

#### 1.4.2.1 Ileal-anal-pouch-anastomosis surgery and pouchitis

Restorative proctocolectomy with ileal-anal-pouch-anastomosis (IPAA) has been the surgical treatment of choice for medically refractory UC since the introduction of the procedure in 1978.<sup>124</sup> The procedure is usually carried out in two stages, initially with complete colectomy and ileostomy, later reversed to incorporate the distal ileum as a reservoir for stool, allowing for restored intestinal continuity.<sup>121</sup> The most common complication to IPAA surgery is pouchitis – inflammation of the pouch. It is reported that 50% of patients that undergo IPAA will develop pouchitis at some point, most commonly within the first year post-surgery.<sup>125</sup> An estimated 5-10% of patients will develop chronic pouchitis, which is the most common reason for pouch failure.<sup>126</sup> The course of pouchitis comprises a wide spectrum of clinical presentations such as increased stool frequency, urgency, incontinence, abdominal pain, bloody stools and fatigue.<sup>127</sup> Diagnosis should be based on a combined assessment of symptoms, endoscopic and histological findings,<sup>128, 129</sup> although the disease is heterogeneous and a mismatch between clinical symptoms and endoscopic findings is not uncommon.<sup>130</sup> While treatment modalities should be individualized, they usually include antibiotics that can relieve symptoms.<sup>129, 131</sup> The therapeutic effects of antibiotic treatment indicate that the gut microbiota is involved in the pathogenesis of pouchitis. After ileostomy closure, the ileal epithelium of the pouch becomes colonic in appearance in accordance with faecal stasis, and the bacterial communities begin to resemble those of the colon, more than those of the ileum.<sup>132</sup> McLaughlin et al. analysed pouch biopsies from patients with IPAA due to UC (UC-IPAA) and familial adenomatous polyposis (FAP), both with and without pouchitis by 16S rRNA gene sequencing. They found a significant increase in *Proteobacteria* spp. in patients with UC but without pouchitis, compared to FAP patients with no pouchitis, and found that bacterial diversity was higher in UC patients without pouchitis compared to those with pouchitis.<sup>133</sup> Microorganisms such as *Clostridium difficile*,<sup>134</sup> *Campylobacter*<sup>135</sup> and Cytomegalovirus<sup>136</sup> have been suspected of being involved in the etiology of pouchitis, but a clear correlation to any specific microorganism has yet to be established. Activity of the adaptive immune system in patients with pouchitis mimics the finding in UC with increased proliferation of inflammatory cells and production of proinflammatory cytokines in the gut mucosa.<sup>127</sup> In general, the development and maintenance of pouchitis resembles the natural course of UC, where an initial acute inflammatory response can become persistent, and patients with pouchitis represent UC patients with the most severe form of disease.

## CHAPTER 2. OBJECTIVES

While research to support the role of certain pathogenic strains of *C. concisus* in IBD is mounting, cultivation of isolates from the gut mucosa has been very limited. The presence of *C. concisus* DNA does not differentiate between viable and transient strains, and isolates for genetic comparison of intra- and interpersonal strain differences are needed in order to elucidate a possible association to enteric disease.

The aims of this thesis were:

- 1) To optimize cultivation procedures of *C. concisus* from gut mucosal biopsies from IBD patients and healthy controls, with the aim of determining the prevalence of viable *C. concisus* in the intestine.
- 2) To evaluate genetic differences in intra- and interpersonal *C. concisus* isolates from stool, saliva and gut mucosal biopsies from IBD patients and healthy controls by using whole genome sequence data.
- 3) To assess the clinical significance of *C. concisus* colonization in the gastrointestinal tract by investigating the existence of virulence associated genes in isolates from IBD patients and healthy controls.



# CHAPTER 3. MATERIALS AND METHODS

## ***3.1. Study participants and inclusion***

Participants for this study were included during the period May 2014 – December 2015, and were adults (>18 years), from North Jutland, Denmark. Participants were either patients with IBD or healthy controls (adults with a family history of colonic cancer or previously identified polyps, with no current symptoms of gastrointestinal disease or persistent diarrhea, and no histological signs of IBD). Patients with IBD were further subdivided by disease phenotype into CD, UC or UC-IPAA. Participants were sent written information about the project when called in for routine colonoscopies, or when a colonoscopy was assigned in relation to ambulant assessment. Participants were excluded if they had received antibiotics in the four weeks prior to inclusion, if they were pregnant or for some reason not able to provide consent. Other exclusion criteria were malignancy, severe immunodeficiency, hemophilia or anticoagulant treatment.

Oral and written consent to participate was obtained prior to the colonoscopy. The study was approved by the Ethics Committee of Northern Jutland (N-20130070). An overview of the patients' baseline characteristics is outlined in Table 2, and detailed clinical information of each participant is presented in Appendix A. The response rate to participate was 37% for IBD patients, and 22% for healthy controls. Two IBD responders were not eligible for participation due to recent antibiotic treatment, and one HC was not eligible due to treatment with anticoagulants. In total, 57 IBD patients (13 CD, 17 UC, 27 UC-IPAA), 28 healthy controls and two patients with persistent diarrhea that had normal colonoscopies were included.

Saliva and stool samples were obtained prior to colonoscopy whenever possible, and mucosal biopsies were sampled from the following anatomical locations: the terminal ileum (TI), cecum (CE), ascending colon (ASC), transverse colon (TRA), descending colon (DES), sigmoideum (SIG) and rectum (REC), when possible. For some patients, strictures due to disease complications or previous surgery prevented biopsies to be collected from all locations. From UC-IPAA patients, mucosal biopsies were collected from the ileum (just proximally from the afferent limb), the proximal (PP) and distal pouch (DP). Biopsies were retrieved with sterile forceps (Medwork, Braun Scandinavia, Denmark) that were discarded after each individual use. Mucosal biopsies were not obtained from five IBD patients (1 UC, 4 CD) due to logistic challenges. Saliva was not obtained from one patient with UC and faecal samples were not collected from one patient with UC, one with UC-IPAA, two with CD and one HC.

**Table 2:** Baseline characteristics of all study participants.

	<b>IBD</b>			<b>HC</b>	<b>GE</b>
<b>Disease</b>	CD	UC	UC-IPAA		
<b>Number</b>	13	17	27	28	2
<b>Sex, male (%)</b>	5 (38)	8 (47)	14 (52)	13 (46)	0 (0)
<b>Age, mean (range)</b>	41 (24-59)	52 (33-69)	44 (21-63)	51 (30-69)	38 (20-57)
<b>Biopsies, median (range)*</b>	7 (6-7)	7 (4-7)	3 (3-3)	7 (7-7)	7 (7-7)

\*Excluding patients where mucosal biopsies were not provided.

### 3.2. Cultivation of *Campylobacter concisus*

#### 3.2.1. Saliva samples

Saliva samples were smeared directly onto 5% blood agar plates with 1% yeast extract (SSI Diagnostica, Denmark), using cotton tip swabs. Two 5 mg vancomycin tablets (Neo-sensitabs™, Rosco Diagnostica A/S, Denmark) were then placed on the plate, which was incubated for two days in a microaerobic (80% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% H<sub>2</sub>, 5% O<sub>2</sub>) atmosphere at 37°C. Microaerobic conditions were attained using the Anoxomat Mart II system (Mart Microbiology B.V., Netherlands) (Figure 5). Following incubation, growth from around the vancomycin tablets was harvested using inoculation loops, and liquefied in 100 µL sterile saline. Three to four drops from this emulsion was then transferred onto polycarbonate filters (0.6 µm pore size) (Whatman® Nuclepore™, Sigma-Aldrich, MO, USA) and incubated at 37 °C for one hour in an ambient atmosphere. Filters were subsequently removed with sterile forceps and the agar plate incubated in microaerobic atmosphere for a total of 96 hours, according to the previously described protocol.<sup>57</sup> Plates were inspected daily and colonies resembling *Campylobacter* species were investigated by wet mount microscopy and identified by mass spectrometry using the Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) (BRUKER DALTONIK GmbH, Bremen, Germany). Identification was also confirmed with qPCR analysis (Life Technologies, Carlsbad, CA, USA) using *C. concisus* specific primers<sup>137</sup> targeting the 16S rRNA gene with modifications of the methods of Mahendran et al.<sup>52</sup>

#### 3.2.2. Stool samples

Study participants were instructed in collecting stool samples in dry containers that were sent by mail to the laboratory on the day of collection, rendering a transit time

of 2-4 days. On receipt in the laboratory, stool samples were kept refrigerated until diagnostic procedures were carried out, which was no later than 24 hours after reception. Two hundred milligrams of faeces was transferred to a clean eppendorf tube before processing, then labelled and stored in a biobank. The remainder of the stool samples were liquefied by addition of saline in volumes equivalent to the faecal mass, and vortexed until the emulsion was homogenous. Five to six drops of the emulsion was then transferred onto polycarbonate filters, incubated and processed as described for saliva samples.



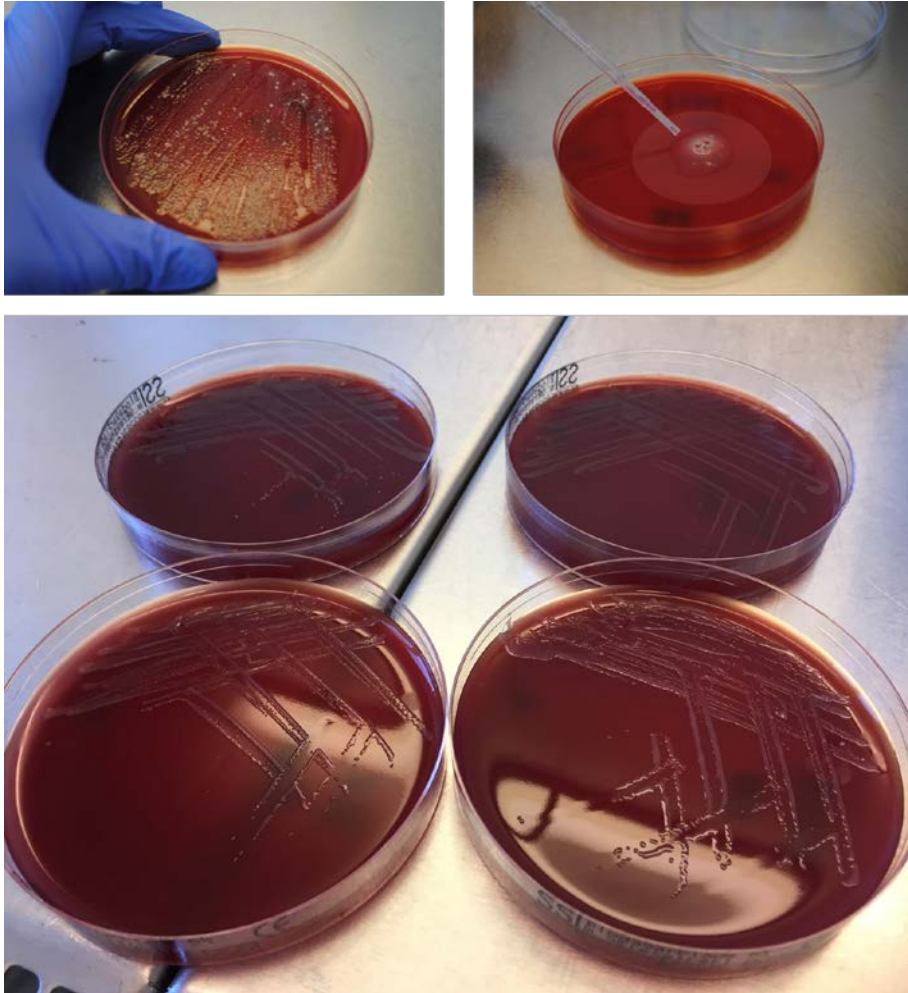
**Figure 5:** The Anoxomat Mart II chamber system. Used for implementing different incubation environments. (Author's own photo).

### 3.2.3. Mucosal biopsies

Tissue samples were promptly placed in sterile containers with 0.5 ml sterile saline, marked with patient ID and location of sampling. Biopsies were then immediately taken to the laboratory, rendering a transit time of less than 30 minutes at ambient temperature. Each biopsy was smeared onto two 5% blood agar plates with added yeast extract using sterile inoculation loops.

Cultivation was then carried out as a two-step procedure: Initially, one plate was incubated for 48 hours in microaerobic and the other in anaerobic (80% N<sub>2</sub>, 10% CO<sub>2</sub>, 10% H<sub>2</sub>) conditions. Following 48 hours of incubation, inoculation loops were used to harvest approximately 100 µg bacterial mass by streaking across each agar plate. The bacterial mass was liquefied by addition of 50 µl sterile saline and brief vortexing. Using sterile pipettes, the emulsion was transferred onto two 5% blood agar plates with added yeast extract and polycarbonate filters, according to the previously described method for saliva and stool samples (Figure 6).

Immediately after the biopsies were smeared onto agar plates for cultivation, they were placed in individual sterile tubes with sterile saline in preparation for DNA extraction. A detailed laboratory protocol for cultivation is presented in Appendix A.



**Figure 6:** Top left: Initial growth from a biopsy smear after two days incubation in micro-aerobic atmosphere, transferred to plates with polycarbonate filters (top right). Bottom: Different phenotypic *C. concisus* cultures. Authors own photos.

### 3.3. Polymerase Chain Reaction (PCR)

#### 3.3.1. Introduction to PCR

In this study, qPCR was performed using the 7500 Real-Time PCR system (Applied Biosystems, Thermo Fischer Scientific, Massachusetts, USA). Standard curves used for quantification were generated by serial dilution of template DNA from a *C. concisus* isolate. A Ct-value of < 35 was considered positive, correlating to a bacterial



concentration of at least  $1 \times 10^2$  CFU/ml. In all PCR runs, a negative template control (NTC) was included. PCR products were visualised on QiaExcel Advanced Screen Gel (Qiagen, Hilden, Germany) for verification of product size using an in-house protocol.

### 3.3.2. PCR methods – Saliva and stool

Extraction of DNA from saliva and stool samples was conducted in an identical manner. Approximately 100  $\mu$ l of sample was mixed with 500  $\mu$ l sterile saline and vortexed until homogeneous. Extraction was performed with the NucliSENS® easyMag® (BioMérieux, Marcy-l'Étoile, France) using the on-board protocol A with a final elution volume of 110  $\mu$ l. The thermal cycling conditions for the *C. concisus* specific qPCR were 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The PCR reaction volume was 25  $\mu$ l. The primers ConcFmod (5'-CAAGTCTCTTGTGAAATCCTATG-3') and ConcR2 (5'-TTACTGCCAAGACTAGCTTAG-3') targeting a 260 bp sequence of the *C. concisus* 16S rRNA gene were used in the reaction.<sup>137</sup>

### 3.3.3. Mucosal biopsies

Immediately following cultivation procedures, mucosal biopsies were placed in 2 ml safe-lock tubes with 500  $\mu$ l sterile saline, 0.3 g glass beads (0.5 mm) and one stainless steel bead (5 mm). The tubes were beaten at 20 Hz for three minutes in a TissueLyser II (Qiagen, Hilden, Germany). Safelock tubes were then centrifuged for 30 seconds, after which the supernatant was transferred to clean eppendorf tubes. DNA extraction was performed as described above for saliva and faecal samples.

DNA extracted from intestinal biopsies were subjected to a two step PCR. The bacterial 16S rRNA gene was first amplified from DNA extracted from intestinal biopsies using universal primers F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R1496 (5'-TACGGCTACCTTGTT ACGAC- 3'), targeting a 1467 bp sequence from bacterial 16S rRNA.<sup>138</sup> The thermal cycling conditions were 95°C for 10 minutes, followed by 35 cycles of 95°C for 20 seconds, 63°C for 20 seconds and 72°C for 1 minute. The PCR reaction volume was 25  $\mu$ l. The PCR product from this reaction was then purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. The purified PCR product (5  $\mu$ l) was subsequently subjected to a *C. concisus*-specific PCR using the primers ConcFmod and ConcR2, described previously. The detailed laboratory protocol and reagents used for all PCR reactions are presented in Appendix C.

### **3.4. Whole-genome sequencing (WGS)**

#### **3.4.1. Introduction to WGS**

The first whole genome to be sequenced was the virus phage  $\phi$ X<sub>174</sub>, by Fred Sanger in 1977.<sup>139</sup> Sanger sequencing is a technology involving chain-termination, sometimes called the “dideoxy technique”. Dideoxyribonucleotides (ddNTPs) are chemical analogues of the deoxyribonucleotides (dNTPs) that lack the 3’ hydroxyl group, required for DNA extension. By adding a small volume of radiolabeled ddNTPs in a normal extension reaction, random incorporation into the copied DNA will result in different strand lengths. The reaction is performed in parallel for each individual ddNTP base (A, C, G, T), and the sequence is then determined by running the results on polyacrylamide gels.<sup>139</sup> Sanger sequencing was modified to incorporate fluorometric based detection and capillary based electrophoresis, which led to the first commercialized sequencing machines.<sup>140</sup> Whole genome sequencing is now routinely used in many clinical microbiology laboratories in identifying pathogens, resistance genes and for epidemiological surveillance of infectious diseases.<sup>141</sup>

#### **3.4.2. DNA extraction for WGS**

In our study, 104 *C. concisus* isolates were sequenced, representing isolates across the sampling frame to capture as much genetic diversity as possible (Appendix D). Isolates were cultivated as previously described and DNA extraction was performed using the QIAamp DNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions and quantified with the Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, Carlsbad, CA, USA), and the Quant-iT DNA Assay Kit (Life Technologies, Paisley, UK), just prior to sequencing. The purity of DNA was assessed by measuring 260/280 OD on a Nanodrop 8000 (Thermo Fischer Scientific, Massachusetts, USA). The mean DNA concentration for the final products was 46.5 ng/μl (range 20-60). The mean 260/280 OD was 2.0 (range 1.56-2.0).

#### **3.4.3. Sequencing method**

Whole genome sequencing was performed using a MiSeq desktop sequencer (Illumina, San Diego, California, U.S.A). The Illumina NGS workflow is based on four steps: Initial DNA extraction and library preparation with DNA fragmentation and ligation of adapters. This is followed by a PCR technique termed bridge amplification, and the resulting clusters are subsequently sequenced by incorporation of fluorescently labelled nucleotides that emit signals corresponding to the different

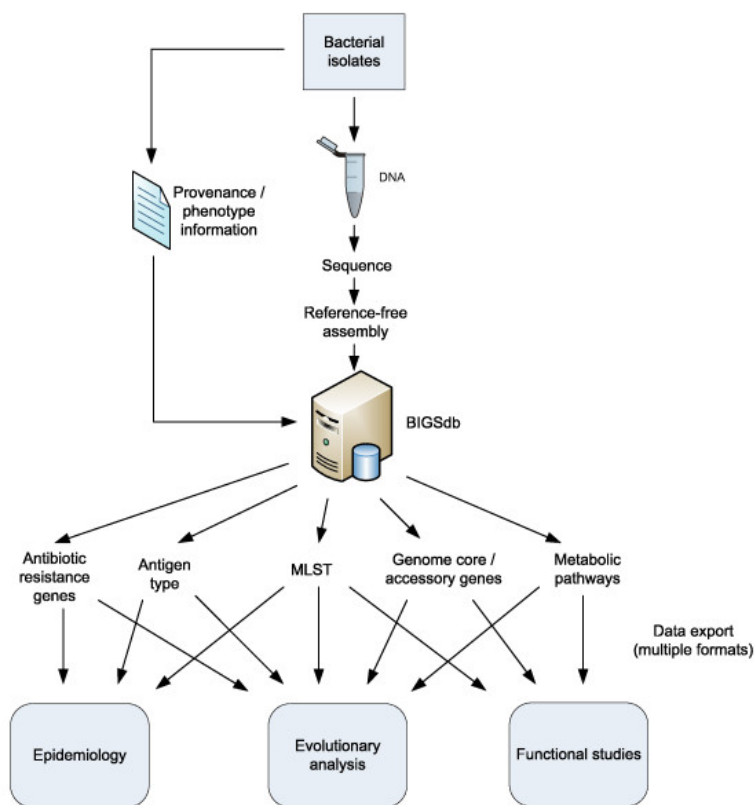
bases. Following sequencing, reads are aligned and assembled with computer based programs, prior to data analysis. (<http://www.illumina.com/techniques/sequencing/dna-sequencing/whole-genome-sequencing.html>).

Genome sequencing was performed on an Illumina MiSeq sequencer using the KAPA Low-Throughput Library Preparation Kit with Standard PCR Amplification Module (Kapa Biosystems, Wilmington, MA), following manufacturer's instructions except for the following changes: Seven hundred and fifty nanograms DNA was sheared at 30 psi for 40 s and size selected to 700–770 bp following Illumina protocols. Standard desalted TruSeq LT and PCR Primers (Integrated DNA Technologies, Coralville, IA) were used at 0.375 and 0.5  $\mu$ M final concentrations, respectively. PCR was reduced to 3–5 cycles. Libraries were quantified using the KAPA Library Quantification Kit (Kapa), except with 10  $\mu$ l volume and 90-s annealing/extension PCR, then pooled and normalized to 4 nM. Pooled libraries were re-quantified by ddPCR on a QX200 system (Bio-Rad), using the Illumina TruSeq ddPCR Library Quantification Kit and following manufacturer's protocols, except with an extended 2-min annealing/extension time. Libraries were sequenced using 2  $\times$  250 bp paired end v2 reagent kit on a MiSeq instrument (Illumina) at 13.5 pM, following manufacturer's protocols. Reads were obtained from SeqWright (Houston, TX). Short read paired-end data was assembled using the de novo assembly algorithm, Velvet (version 1.2.08).<sup>142</sup> The VelvetOptimiser script (version 2.2.4) was run for all odd k-mer values from 21 to 99. The minimum output contig size was set to 200 bp with the scaffolding option switched off; all other program settings were left unchanged. The average number of contigs was 92 (range: 3-356) for an average total assembled sequence size of 1.94 Mbp (range: 1.78-2.22). The average N50 was 97693 (range: 13858-934037) and the average GC content was 38.94% (range: 37.26-39.88). Contigs were individually archived in sequence bins on the web-based platform BIGSdb.<sup>143</sup>

Assembled genome data and individual genome coverage information is presented in Appendix E, along with corresponding BIGSdb ID's. Genomes are pending submission to the NCBI database.

### 3.4.4. Data analysis – the Bacterial Isolate Genome Sequence Database (BIGSdb)

The Bacterial Isolate Genome Sequence Database (BIGSdb) is a web-accessible database that holds sequence information for thousands of bacterial isolates.<sup>143</sup> The sequence information is linked to isolate records and metadata, and can be used for genome comparison and BLAST searches for specific gene content (Figure 7). Data is exported as tab-delimited text files or concatenated sequences in FASTA format for use in various analyses.



**Figure 7:** The BIGSdb workflow. From: Jolley & Maiden, *BMC Bioinformatics* 2010, 11:595. Reprinted under the Creative Commons attribution licence (CC-BY).

Orthologous genes were defined as homologous genes that had  $\geq 70\%$  nucleotide identity and  $< 50\%$  difference in alignment length (default settings).

### 3.5. Multi-Locus sequence typing (MLST)

Multi-locus sequence typing was introduced as a method for typing of bacterial microorganisms in a way that information could be exchanged between laboratories and used for epidemiological purposes.<sup>144</sup> In MLST schemes, a number of housekeeping genes (usually at least seven) are sequenced and each assigned an allele number. The combined allelic profile is then assigned a sequence type (ST). The assignment of a unique allele disregards how many nucleotide polymorphisms are involved. MLST allele sequences and ST profile tables for bacterial species are stored in online databases, and can be collectively accessed from [www.pubmlst.org](http://www.pubmlst.org), hosted by the Department of Zoology at the University of Oxford.

We used the MLST scheme defined by Miller et al., which targets the seven loci: aspartase (*aspA*), ATP synthase subunit alpha (*atpA*), glutamine synthetase (*glnA*), citrate synthase (*gltA*), serine hydroxyl methyl transferase (*glyA*), dihydroxy-acid dehydratase (*ilvD*) and phosphor glucomutase (*pgm*).<sup>145</sup> For comparison, we also used the scheme described by Ismail et al., using the six housekeeping genes validated for MLST of *C. jejuni* that also include *aspA*, *glnA* and *atpA*, as well as glucose-6-isomerase (*pgi*), transketolase (*tkt*) and aspartate-semialdehyde dehydrogenase (*asd*).<sup>146</sup>

Sequences of housekeeping genes of *C. concisus* isolates were aligned and compared in BIGSdb, and phylogenetic trees were generated using the MEGA7 tool by applying the neighbor-joining method with 500 bootstrap replications.<sup>147</sup> *Campylobacter curvus* 525.92 (Accession No. CP000767.1) was used as an outgroup. For each housekeeping loci, the different sequences were assigned distinct allele numbers. The combination of allelic profiles is pending ST assignment and isolates with identical STs were defined as the same strain.

### 3.6. Zot and Exotoxin 9 BLAST searches

The DNA sequence for the *zot* and exotoxin 9/ DnaI gene were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), and aligned by BLAST in BIGSdb as described above. Nucleotide sequences, polymorphisms and amino acid alignments were visualized using the online BioEdit software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>.)

Primers used for these analyses are listed in table 3.

**Table 3:** List of primers used for *zot* and *exotoxin 9* detection

Target	Name	Sequence (5'→3')	Size* (bp)	Ref.
<b>Zot</b>	FCCC13826_2075	TGCAAACCCTTTGTGATGAA	1055	86
	Ccon_zotR_257	TCGGTCCTCCACGATCTG		148
	Zot1	GCAACTTAGAAAAAGTATCGG	790	72
	Zot2	TAATAGTTCTCGATGAAGCC		72
	ZotF	CTAGAATCAGTTTGTGGAGAT		72
<b>Exotoxin 9</b>	Exotox-F	GAGACAAAGCTGCTTTAT	291	149
	Exotox-R	CTATCAAGATTAAAGCCG		

\* Size of target sequence.

### 3.7. Statistical analysis

Data was analysed using Stata 14 (Statacorp LP, Texas, USA). Where applicable, the  $\chi^2$  test was used for dichotomous variables and McNemar's test for paired nominal data. A p-value < 0.05 was considered statistically significant. When comparing groups in study II and III, comparison was calculated between the number of patients rather than the number of isolates, i.e. a patient was several positive isolates from the same location counted as only one, since the number of isolates from each individual varied.

# CHAPTER 4. RESULTS

## 4.1. Study I

Patients that did not provide gut mucosal biopsies were not included in the first study, and neither were patients with diarrhea. One of the healthy controls in the total population was not included until after study I was complete, resulting in 78 subjects in total, included in study I. (CD=9, UC=16, UC-IPAA=27, HC=26).

### 4.1.1. Cultivation from Gut Mucosal Biopsies

In total, 427 biopsies were collected from the 78 subjects. The number of biopsies collected at each anatomical site from the different disease categories is presented in Table 4.

**Table 4:** Number of biopsies attained at each location for the different patient groups.

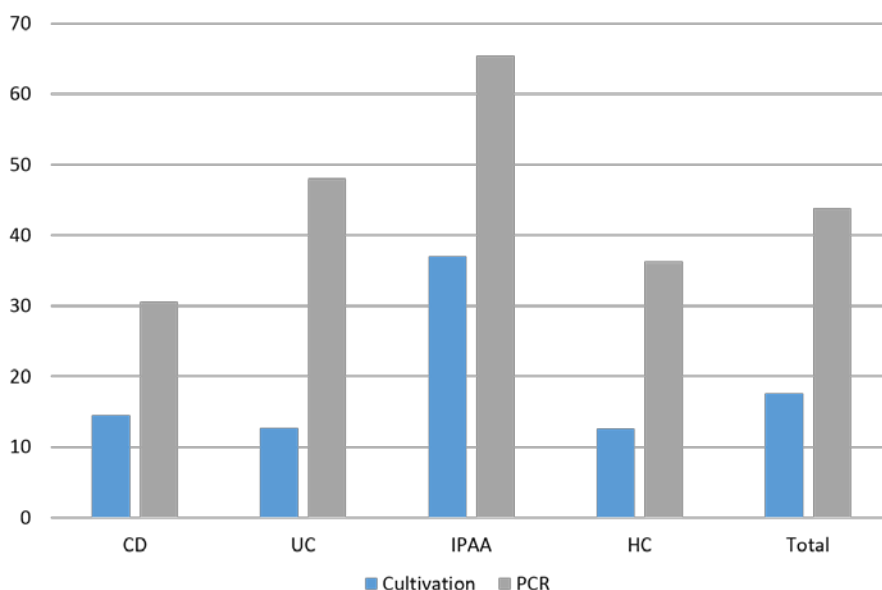
	IBD			HC
Subgroup	CD	UC	UC-IPAA	
Number of participants	9	16	27	26
Terminal ileum	8	9	-	26
Cecum	9	14	-	26
Ascending colon	9	15	-	26
Transverse colon	9	16	-	26
Descending colon	9	16	-	26
Sigmoideum	9	16	-	26
Rectum	9	16	-	26
Ileum (UC-IPAA)	-	-	27	
Proximal pouch (UC-IPAA)	-	-	27	
Distal pouch (UC-IPAA)	-	-	27	
Total	62	102	81	182

Isolates that grew in both microaerobic and anaerobic atmospheres were collected separately, rendering a total of 99 isolates. Twenty-nine isolates were derived from microaerobic and 22 from anaerobic incubation exclusively ( $p=0.40$ ). From 24 biopsies, isolates grew in both atmospheres (Table 5).

**Table 5:** Number of isolates collected from microaerobic and anaerobic cultivation

Incubation method	IBD (%)	HC (%)
Microaerobic only	18 (25)	11 (39)
Anaerobic only	17 (24)	5 (18)
Both	36 (51)	12 (43)
Total	71	28

In all IBD patients, a total of 52/245 (21%) biopsies were culture positive for *C. concisus*, while 121/245 (49%) were PCR positive ( $p<0.001$ ). For healthy controls, the numbers were 23/182 (13%) and 66/182 (36%), respectively ( $p<0.001$ ). The rate of cultivation and PCR detection was higher for IBD patients compared to healthy controls ( $p=0.021$  and  $p=0.008$ , respectively), but interestingly, it was significantly higher for UC-IPAA patients compared to other IBD patients ( $p=0.0001$  and  $p=0.0006$ , respectively) (Figure 8).

**Figure 8:** Percentage of positive biopsies (in total) by cultivation and PCR from the different patient groups.

Agar plates were also inspected for *Campylobacter* species other than *C. concisus*. The majority of these were *C. ureolyticus*, with six isolates recovered from six different IBD patients. One of these patients was also positive for *C. concisus*. One IBD patient had *C. curvus* in three different biopsies; another had one *C. showae*

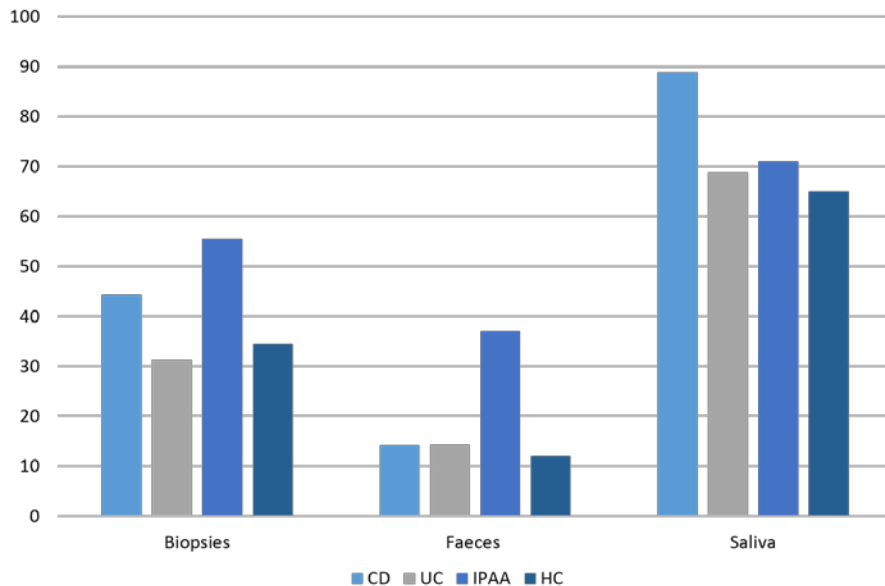


isolate in one biopsy. Both of these patients were also positive for *C. concisus*. In the healthy control group, isolates of *C. ureolyticus* and *C. showae* were recovered from four different individuals, whereof two were also positive for *C. concisus*. No thermophilic *Campylobacter* species were isolated from IBD patients or healthy controls.

#### 4.1.2. Cultivation of saliva and stool samples

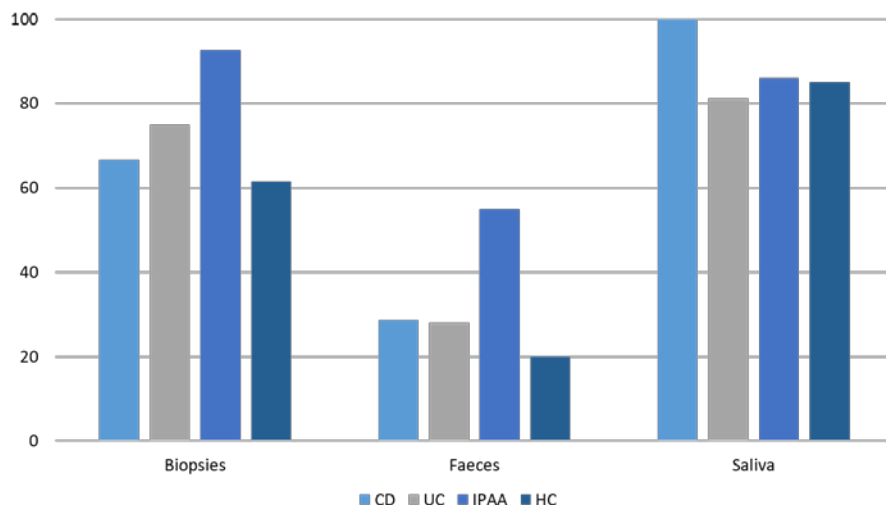
From stool samples, 12/48 (25%) isolates from IBD patients were culture positive, while 19/48 (40%) were positive by PCR detection. For healthy controls, the numbers were 3/25 (12%) and 5/25 (20%) respectively. There was no difference between groups by cultivation ( $p=0.18$ ), but a significantly higher amount of positive stool samples by PCR in the UC-IPAA subgroup ( $p=0.001$ ). As expected, *C. concisus* was abundant in saliva samples from both IBD patients and healthy controls, with 34/51 (67%) IBD and 18/26 (70%) healthy controls being culture positive, while 47/51 (92%) and 23/26 (88%) were PCR positive, respectively.

When assessing the number of subjects with at least one positive *C. concisus* biopsy, stool or saliva sample, there was no difference between groups by cultivation (biopsies  $p=0.26$ , faeces  $p=0.12$ , saliva  $p=0.60$ ) (Figure 9).



**Figure 9:** Percentage of individuals with at least one culture positive *C. concisus* sample from biopsies, faecal samples and saliva.

When assessing PCR results, there was no difference in the number of people with at least one positive biopsy ( $p=0.06$ ) or saliva sample ( $p=0.60$ ), but just marginally, there were more UC-IPAA patients with a positive faecal sample ( $p=0.05$ ) compared to the other groups. (Figure 10).



**Figure 10:** Percentage of individuals with at least one PCR positive *C. concisus* sample from biopsies, faecal samples and saliva.

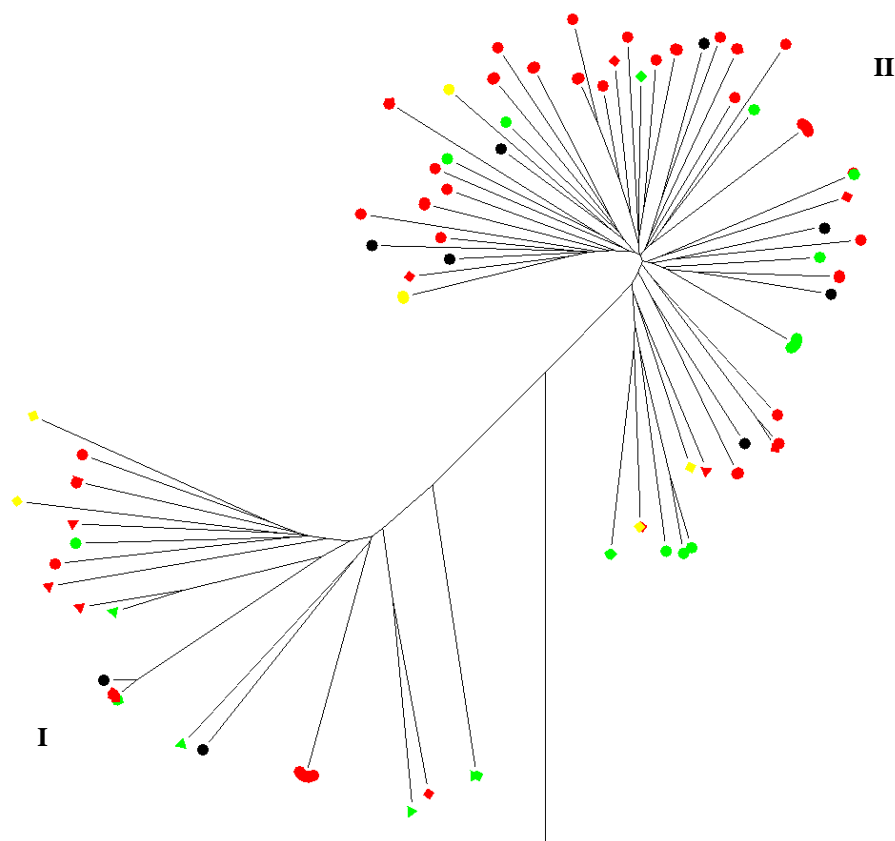
When assessing clinical symptoms of the IBD patients in the study, there was significantly more UC-IPAA patients with clinical symptoms compared to CD patients and other UC patients ( $p=0.03$ ). Clinical symptoms were diarrhea, blood or mucus in stool, abdominal pain, or fatigue, and were marked as present if the symptoms were worse than the patient's usual status at baseline. Interestingly, the presence of endoscopic (ulcerations, edema, friability) and histological (polymorph infiltration, ulcerations) inflammation was not significantly different between groups (Table 6).

**Table 6:** Number of IBD patients with clinical symptoms, endoscopic and microscopic signs of inflammation.

	CD	UC	UC-IPAA	p-value
Clinical symptoms (%)	5/9 (56)	7/16 (44)	22/27 (82)	0.03
Endoscopic inflammation (%)	4/9 (44)	7/16 (44)	18/27 (67)	0.26
Microscopic inflammation (%)	5/9 (56)	7/16 (44)	21/27 (78)	0.07

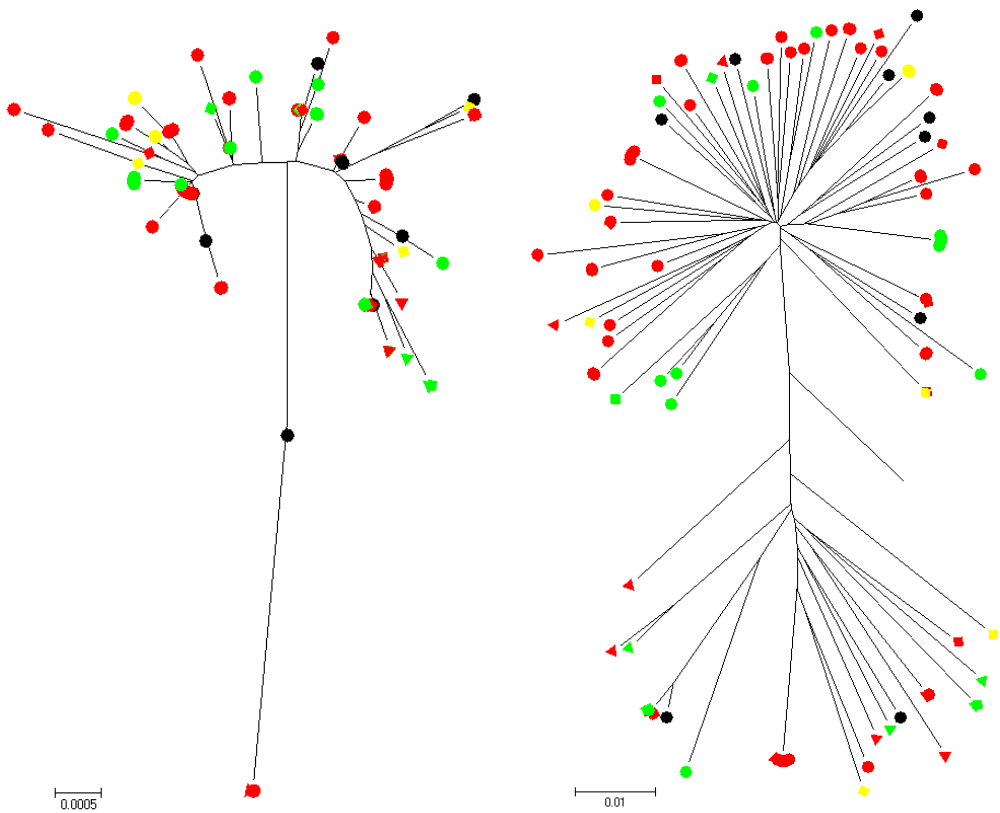
## 4.2. Study II: MLST

Multi locus sequence typing using the combination of loci defined by Miller et al. revealed a high diversity of *C. concisus* with 71 ST's and the following number of alleles: *aspA*:63, *atpA*:65, *glnA*:62, *gltA*:64, *glyA*:62, *ilvD*:64 and *pgm*:63. The total length of the combined sequences was 3345 bp. For one isolate, the *ilvD* loci was located at the end of a contig and the allele therefore incomplete. The *atpA* loci was not present in the *C. curvus* 525.92 genome. *C. concisus* isolates clustered into two main groups corresponding to 23S rRNA genomospecies (GS I and GS II), with the majority of isolates in GS II (n=79). There was no association to disease status, as IBD patients, diarrheic patients and healthy controls were represented in both clusters (p=1.00). However, when assessing anatomical site of collection, there was significantly more mucosal isolates in GS II and oral isolates in GS I. (p<0.0001). Faecal isolates were equally present in both clusters (Figure 11).



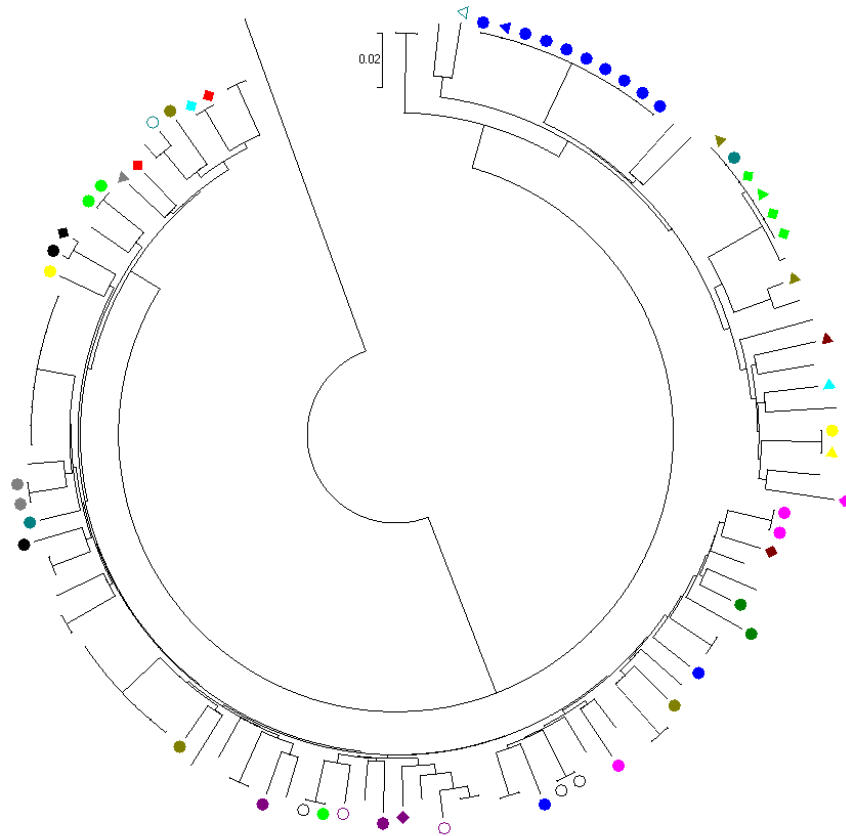
**Figure 11:** Neighbour-joining tree from concatenated MLST sequences of *C. concisus* isolates, displayed in radiation for visual interpretation of clustering into two groups. The colours represent disease state: Red = IBD, yellow = GE, green = HC. Shapes indicate site of sampling: circles = mucosal biopsies, diamonds = faecal isolates, triangles = saliva.

As previously mentioned, Deshpande and colleagues found that typing by 16S rRNA sequences showed a division of isolates that correlated to clinical presentation in the eight genomes sequenced in that study.<sup>73</sup> In the isolates from our study, we found no correlation to disease presentation or sampling site when comparing 16S rRNA sequences. (Figure 12, left). We also assessed the MLST scheme presented by Ismail and colleagues, and found that clustering was identical to the findings when using the scheme of Miller et al., with 71 unique strains (Figure 12, right). The results discussed in the following are from MLST using the Miller scheme (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *ilvD* and *pgm*), as this is the accepted scheme for entry into the Pubmlst database.



**Figure 12.** Left: Neighbour-joining dendrogram based on 16S rRNA sequences. Presented in radiation forms for visualization purposes. Right: Neighbour-joining dendrogram based on sequences from the six housekeeping loci described by Ismail et al.<sup>146</sup>. Colours and shapes represent disease states and sample location as described in figure 11.

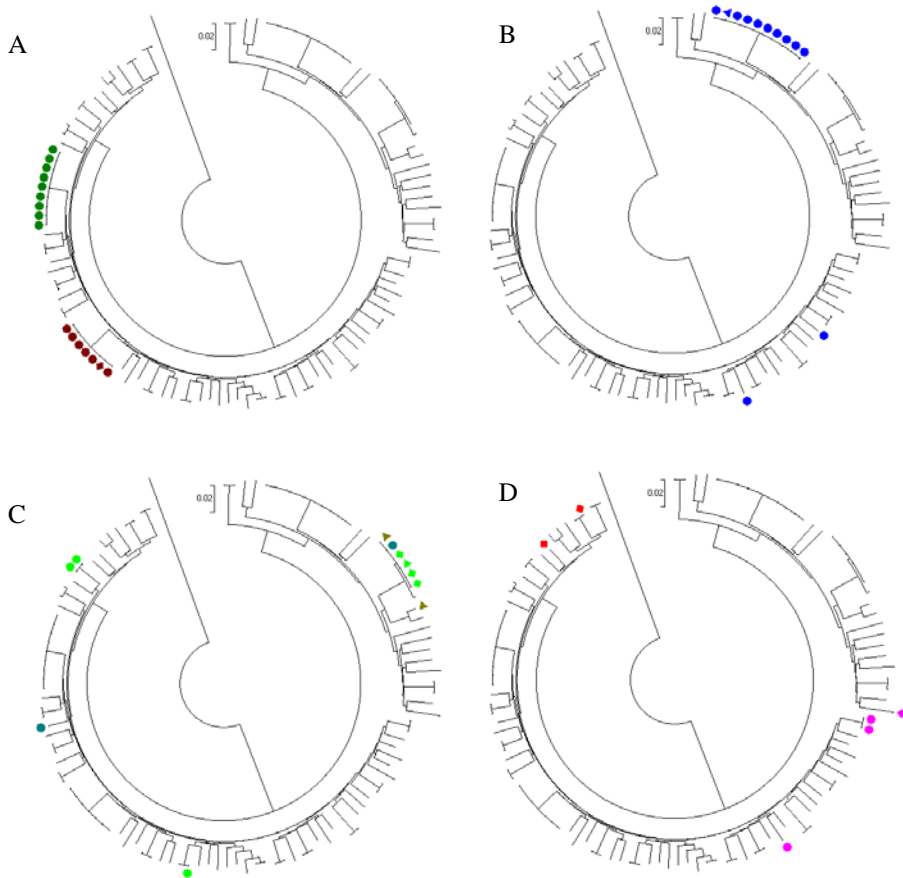
Two or more isolates were collected from 27 patients (18 IBD, 2 GE, 7 HC). Isolates from 17 of these 27 patients (63%) were genetically different, with isolates from seven individuals (4 IBD, 1 GE, 2 HC) being represented in both GS. These findings were independent of clinical presentation and sampling site (Figure 13).



**Figure 13:** Circular dendrogram of concatenated MLST sequences of *C. concisus* isolates, displaying individual variation. The colours represent different individuals, the shapes indicate sampling location as in figure 11 (see text for details).

Two individuals had multiple isolates that were the same ST (Patient 35UC and 10HC) (figure 14a). However, other patients with multiple isolates had more than one ST. Patient 12CD had several mucosal isolates and an oral isolate in the same ST in GS I, but two mucosal isolates with different STs in GS II. One isolate from GS I and an isolate from GS II were retrieved from the same biopsy (from the transverse colon) (Figure 14b). Interestingly three faecal and one oral isolate from patient (16UC (UC-IPAA)), one oral isolate from a healthy control (8HC) and one mucosal isolate from a healthy control (9HC) were all the same ST. However, the mucosal isolates from patient 16UC belonged to GS II (figure 14c).

For some patients, isolates were collected at different time points: The faecal samples from the previously mentioned patient with UC-IPAA (16UC) were collected one month apart, but were all the same ST. Interestingly; one patient (1D) with diarrhea had three different isolates, represented in both GS. The faecal isolate was collected in 2012, and the two biopsy samples in 2015. This patient presented with intermittent episodes of prolonged diarrhea, but had no endoscopic or microscopic signs of mucosal inflammation. Another patient with diarrhea (25D) had two different faecal isolates in the same GS. These samples were collected two months apart and the patient was treated with ciprofloxacin in between sample collection (Figure 14d).



**Figure 14:** Circular dendrograms displaying the distribution of a subset of different patients with several isolates. Top left (a): Patients 35UC (brown) and 10HC (green). Both patients only have isolates belonging to the same ST. Top right (b): Patient 12 CD. Bottom left (c): Patients 16 UC (bright green), 8HC (brown), 9HC (blue). Bottom right (d): Diarrheic patients 1D (pink) and 25D (red).

### 4.3. Study III: Putative virulence factors *Zot* and Exotoxin 9

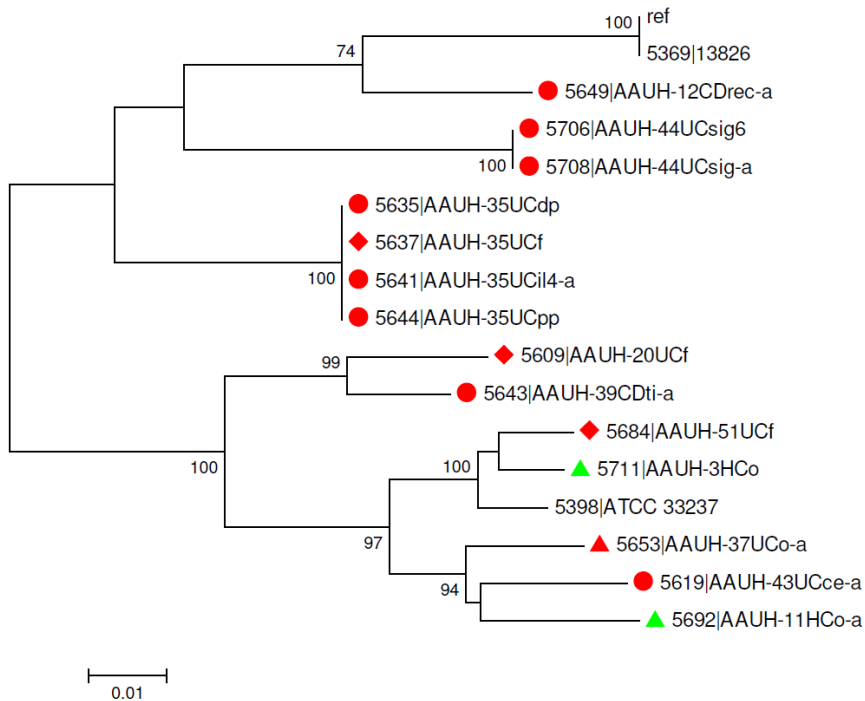
BLAST searches for the *zot* gene and exotoxin 9 (used as a proxy for the UNSWCD plasmid) revealed that 67/104 (64.4%) isolates were positive for either *zot* or exotoxin 9 DNA, or both. Eight isolates were positive for *zot* only, 50 for exotoxin 9 only, and nine had both *zot* and exotoxin 9 (Table 7).

**Table 7:** Isolates positive for *zot* and/or exotoxin 9 and corresponding patient information

Individual	Disease Phenotype	No. of isolates	<i>C. concisus</i> isolates
4UC	UC	2	AAUH-4UCti (B), AAUH-4UCti_a (B)
7UC	UC-IPAA	1	AAUH-7UCil (B)
8UC	UC-IPAA	1	AAUH-8UCo (O)
11UC	UC	1	AAUH-11UCsig_a (B)
12CD	CD	1	AAUH-12CDrec_a (B)
15UC	UC-IPAA	1	AAUH-15UCdp (B)
16UC	UC-IPAA	6	AAUH-16UCf (F), AAUH-16UCf2 (F), AAUH-16UCf3 (F), AAUH-16UCo_a (O), AAUH-16UCdp3 (B), AAUH-16UCdp5 (B)
20UC	UC-IPAA	1	AAUH-20UCf (F)
35UC	UC-IPAA	7	AAUH-35UCdp (B), AAUH-35UCil_a (B), AAUH-35UCil2_a (B), AAUH-35UCil3_a (B), AAUH-35UCil4_a (B), AAUH-35UCpp (B), AAUH-35UCf (F)
37UC	UC-IPAA	2	AAUH-37UCo (O), AAUH-37UCf (F)
39CD	CD	3	AAUH-39CDf (F), AAUH-39CDti_a (B), AAUH-39CDrec_a (B)
40UC	UC	1	AAUH-40UCf (F)
43UC	UC	1	AAUH-43UCce-a (B)
44UC	UC	2	AAUH-44UCsig_a (B), AAUH-44UCsig6 (B)
47UC	UC-IPAA	2	AAUH-47UCil (B), AAUH-47UCil_a (B)
48UC	UC-IPAA	1	AAUH-48UCdp_a (B)
49UC	UC-IPAA	3	AAUH-49UCil_a (B), AAUH-49UCpp_a (B), AAUH-49UCf (F)
51UC	UC-IPAA	1	AAUH-51UCf (F)
59UC	UC-IPAA	1	AAUH-59UCpp_a (B)
2HC	HC	1	AAUH-2HCtra (B)
3HC	HC	2	AAUH-3HCo (O), AAUH-3HCco (B)
6HC	HC	1	AAUH-6HC0_a (O)
8HC	HC	1	AAUH-8HCo (O)
9HC	HC	2	AAUH-9HCasc (B), AAUH-9HCco (B)
10HC	HC	9	AAUH-10HCco (B), AAUH-10HCtra (B), AAUH-10HCdes (B), AAUH-10HCdes2 (B), AAUH-10HCdes3 (B), AAUH-10HCdes4 (B), AAUH-10HCdes5 (B), AAUH-10HCdes6 (B), AAUH-10HCdes7 (B)
11HC	HC	2	AAUH-11HCo (O), AAUH-11HCf (F)
12HC	HC	1	AAUH-12HCf (F)
14HC	HC	1	AAUH-14HCco (B)
15HC	HC	1	AAUH-15HCti (B)
20HC	HC	1	AAUH-20HCrec_a (B), AAUH-20HCsig_a (B)
1D	GE	3	AAUH-1Dasc (B), AAUH-1Dtra (B), AAUH-1Dce_a (B)
5676	GE	1	AAUH-2010376221
25D	GE	2	AAUH-25Df (F), AAUH-25Df3 (F)

Isolate origin: O: oral, B: biopsy, F: faeces. Blue=*zot* positive, green=exotoxin 9 positive, red=*zot* and exotoxin 9 positive. Reference strains are not presented in this table.

Several individuals had more than one *C. concisus* isolate, sometimes from different locations. In total, 17 out of 104 (16%) isolates were positive for *zot*, from 12 different patients (IBD n=8, HC n=3, GE n=1). The *zot* gene has previously been described in the reference isolate *C. concisus* 13826 (ATCC BAA-1457) and was in this study also detected in ATCC 33237. These sequences were included in the phylogenetic analysis, which showed grouping into two main clusters. No individual had more than one type of *zot*, regardless of sampling site (Figure 15). Three isolates were not included in the phylogenetic analysis because the genes were located at the end of a contig and therefore incomplete (AAUH-2010376221 (faecal isolate, diarrhetic patient), 14HC (mucosal isolate, healthy control) and 11HC (faecal isolate, healthy control)).



**Figure 15:** A Phylogenetic tree based on the concatenated sequences of the *zot* gene in 14 clinical *C. concisus* isolates and 2 reference isolates (13826 and ATCC 33237). Isolates deriving from IBD patients are marked with red, isolates from healthy controls in green. The shapes indicate sampling site: Circles = mucosal biopsies, diamonds = faecal samples and triangles = saliva. The numbers to the left of isolate names are the corresponding BIGSdb id's.

When analyzing nucleotide sequence data and amino acid composition, we found that none of our isolates had the *zot*<sup>350-351AC</sup> polymorphism previously described by Mahendran et al.<sup>86</sup> The *zot*<sup>multiple</sup> polymorphism was detected in three isolates (13826, 44UCsig6 and 44UCsig-a). We found the *zot*<sup>808T</sup> mutation only in one mucosal isolate,



interestingly, from a healthy control (14HC). The amino acid substitutions from the polymorphism sites were equivalent to those previously reported by Mahendran et al., with a substitution of valine at position 270.<sup>86</sup> We did not find any other unique polymorphisms or amino acid substitutions in our *zot* sequences that correlated to clinical presentation.

In total, 59 (56.7%) isolates from 26 different patients (IBD n=15, HC n=9, GE n=2), had exotoxin 9 DNA. There was noticeably fewer isolates with exotoxin 9 only from IBD patients (37.1%) compared to HC (70.4%) and GE patients (71.4%). Nine isolates were positive for both *zot* and exotoxin 9 DNA, and all these nine isolates were from IBD patients, with the majority (n=6) originating from mucosal isolates. An overview of the number of *zot*, exotoxin 9 and combined positive isolates by disease phenotype is presented in table 8, and the number of positive isolates grouped by sampling site, in table 9.

**Table 8:** The number of isolates positive for *zot*, exotoxin 9 and both, for each patient group.

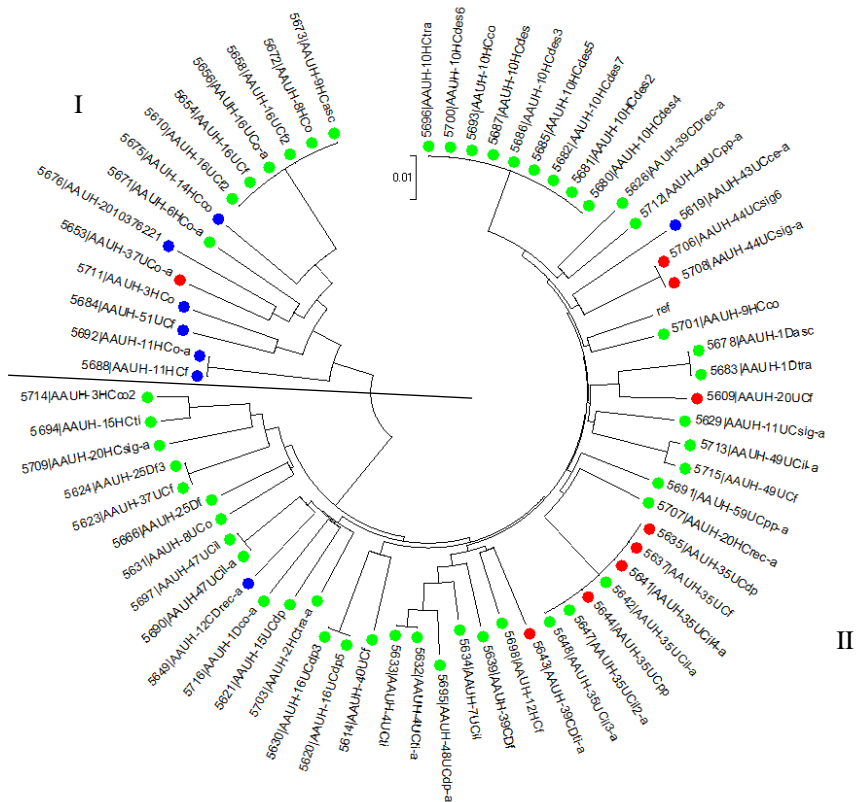
Factor	IBD (n=70)	HC (n=27)	GE (n=7)
<i>zot</i> only	3 (4.3%)	4 (14.8%)	1 (14.3%)
Exotoxin 9 only	26 (37.1%)	19 (70.4%)	5 (71.4%)
<i>zot</i> + Exotoxin 9	9 (12.8%)	0 (0%)	0 (0%)

**Table 9:** The number of isolates positive for *zot*, exotoxin 9 and both, by sample location.

Factor	Saliva (n=14)	Biopsy (n=70)	Stool (n=20)
<i>zot</i> only	2 (14.3%)	3 (4.3%)	3 (15%)
Exotoxin 9 only	4 (28.6%)	36 (51.4%)	10 (50%)
<i>zot</i> + Exotoxin 9	1 (7.1%)	6 (8.6%)	2 (10%)

Interestingly, when assessing the number of virulence genes in each GS, we found that the number of patients with isolates positive for *zot* only, were more prevalent in GS I (6/18) compared to GS II (2/39) ( $p=0.039$ ), whereas the number of patients with isolates positive for exotoxin 9 only, was higher in GS II (20/39) compared to GS I (4/18) ( $p=0.004$ ). The number of individuals with isolates positive for both virulence genes was not significantly different between the two GS (GS I: n=1, GS II: n=4) ( $p=0.56$ ).

When assessing our dataset, it was apparent that simultaneous colonization with isolates both with and without virulence determinants was possible. One patient with Crohn's disease (12CD) had 12 isolates in total. The majority of these, belonging to GS I had neither *zot* nor exotoxin 9, but one of the isolates, from GS II, was positive for *zot*. This isolate was recovered from a mucosal biopsy of the rectum, which did not differ from the other mucosal isolates in terms of macroscopic or histological signs of inflammation at the time of sampling. Seven isolates recovered from a patient with UC-IPAA (35UC) all had exotoxin 9 DNA, and interestingly, four also had *zot*. Some of these isolates were from the same biopsy. From one HC (8HC) two oral isolates were recovered of which one was positive for exotoxin 9 DNA while the other was not. One patient with UC-IPAA (37UC), and one HC (3HC) had an oral isolate with *zot* and a faecal isolate with exotoxin 9, belonging to two different GS. An overview of isolates in each GS with concordant virulence determinants is depicted in figure 16.



**Figure 16:** Circular dendrogram based on the concatenated sequences of seven housekeeping genes of *C. concisus* isolates with detected virulence genes. Isolates marked with blue are positive for *zot*, green for exotoxin 9 and red for both. Roman numerals indicate GS, that are separated by the black line.

# CHAPTER 5. DISCUSSION

## 5.1. Methodological considerations

### 5.1.1. Cultivation

Cultivation from saliva samples using the filter method is relatively simple, and yields a high rate of *C. concisus* from microaerobic cultivation with hydrogen. The rate of *C. concisus* positive faecal samples by cultivation is much lower. In this study, patients were asked to collect faecal samples prior to the colonoscopy procedure. The majority of faecal samples were sent to the laboratory by mail, and were therefore 2-4 days in transit. A limitation to this sampling method is that the samples can be exposed to very high or low temperatures according to variable seasonal weather conditions, which could have an effect on the viability of *C. concisus* isolates. It is also possible that metabolites and waste products from other microorganisms in faecal samples could contribute to degradation of viable bacteria or bacterial DNA. Kaakoush et al. found a higher number of PCR positive faecal samples in a study examining diarrheic isolates of *C. concisus* from children with CD and HC (25/30 (83.3%) and 23/30 (76.7%), respectively).<sup>150</sup> These faecal samples were snap-frozen immediately until use in the study, possibly leading to better DNA conservation.

Cultivation of *C. concisus* from mucosal biopsies has previously been only sparsely described. In some of the first studies to propose an association to IBD, biopsies were cultured on agarplates with added trimetoprim and vancomycin, then transferred onto polycarbonate filters and incubated for 48 hours.<sup>50, 52</sup> Kaakoush and colleagues used an enrichment-filtration procedure in which tissue samples were placed in Ham's F-12 enrichment broth prior to filtration onto agar plates. Using this method, they managed to collect three isolates from three out of 11 children with CD.<sup>149</sup> The main differences in our study design, is that we used several biopsies from different intestinal locations, and combined cultivation in microaerobic and anaerobic atmospheres. While this "Aalborg two-step" method yields a high cultivation rate and facilitates growth, it is space consuming and tedious and therefore not suitable for application in a clinical context. We did not use enrichment broths for tissue samples, but this could be an interesting approach to enhancing *C. concisus* growth possibly reducing the need for multiple sample collection and allowing more time from sample collection to lab processing. We also used non-selective agar plates without added antibiotics. The rationale for using trimetoprim and vancomycin in agarplates is that they limit growth of gram-positive organisms and other gram-negative bacteria such as *E. coli* and *Proteus* species that may make it more difficult for *C. concisus* to grow. However, in our study, we did not encounter cultivation difficulties such as swarming or excessive growth from other organisms, indicating that non-selective medias are sufficient for *C. concisus* cultivation.

There are some basic environmental conditions that were not tested in our study, which could be useful in aiding the optimal cultivation procedures for *C. concisus*. Some outstanding questions that should be addressed are for example, in which temperature range *C. concisus* can grow. While *C. jejuni* has optimal growth conditions in the temperature range 30-46 °C, it maintains vital cellular functions such as ATP generation and protein synthesis at temperatures down to 4°C.<sup>151</sup> Such investigations for *C. concisus* would also be valuable in assessing the possibility that *C. concisus* has an environmental reservoir. Another question is whether or not different enrichment broths could aid in initial growth of *C. concisus* and possibly enhance cultivation rates from clinical samples. As previously mentioned, Kaakoush et al. used the Ham's F12 nutrient broth in cultivation of *C. concisus* from mucosal biopsies,<sup>149</sup> and have previously also described growth in Brain Heart Infusion (BHI)<sup>80</sup> and Bolton broth, with increased growth rates in medias supplemented with horse blood.<sup>76</sup> In a clinical context, investigations into the survival of *C. concisus* in routine transport medias such as the modified Cary-Blair media used in FaecalSwabs™ (Copan Diagnostics, Murrieta, CA, USA) remain to be investigated.

While the studies in this thesis have focused on *C. concisus* in enteric disease, other non-*jejuni*/non-*coli* Campylobacters have also been proposed as potential pathogens of the gastrointestinal tract, especially *C. ureolyticus* and *C. showae*.<sup>51, 53, 54</sup> We managed to culture *C. ureolyticus*, *C. curvus* and *C. showae* from different sample types, but at much lower rates than *C. concisus*. Although we did not perform PCR for other *Campylobacter* species, our results indicate that *C. concisus* is more abundant in the lower gastrointestinal tract than other emerging *Campylobacter* species that have the same nutritional growth requirements.

### 5.1.2. Whole-genome sequencing

Genomic analysis of bacterial isolates by WGS is becoming increasingly available in the clinical setting and will likely replace current technologies for bacterial typing in the near future. Currently, there is no “golden standard” for conducting or reporting on bacterial WGS, and there are several limitations to the analysis that must be considered. Throughout the WGS process from DNA extraction to data analysis, the quality of the in/output must be assessed and reported since analysis depends on the quality of the sequence.<sup>152</sup> In a clinical context, user-friendly bioinformatics pipelines are becoming increasingly available, and this may streamline and uniform the WGS process and create more transparent results.

For WGS in this study, we used the Illumina MiSeq (Illumina, San Diego, California, U.S.A) sequencer, which has a high accuracy and a moderate throughput. A limitation of this sequencing method is the relatively short reads (≈2 x 250 bp) that result in subsequent genome “gaps”. Presently, the PacBio RS II (Pacific Biosciences, Menlo Park, California, USA) sequencer produces the longest reads with a higher resolution

and the possibility of complete closed genomes, but this method has a higher individual read rate error and is currently rather expensive.<sup>152, 153</sup>

### 5.1.3. MLST

Multi-locus sequence typing is a method suitable for data exchange between laboratories, and for describing lineages or clonal complexes. However, differentiation by MLST does not have enough discriminatory power to compare isolates that have undergone microevolution, since only housekeeping genes are compared.<sup>154</sup> However, due to the standardized implementation of MLST schemes, this typing method is reproducible and easily comparable to isolates from other origins, which more in-depth methods like single nucleotide polymorphism (SNP) typing currently is not.<sup>155</sup> In this study, MLST results were sufficient for revealing that vast genetic differences exist not only between individuals, but also within an individual, with some patients having multiple isolates belonging to different ST's. We found that MLST could divide isolates into two GS similar to previous findings by 23S rRNA typing,<sup>148</sup> but this genetic distinction was not associated to disease state. As exemplified in study III, there are genetic differences between isolates of the same ST that could be clinically relevant, and a higher resolution must be implemented in order to differentiate such strains. Whole-, core- and accessory genome MLST of our isolates is currently ongoing.

Our study indicates that human colonization with different *C. concisus* strains is common. Presently, our knowledge about transmission and duration of *C. concisus* colonization is very limited. While colonization of the human oral cavity suggests human to human transmission, *C. concisus* has also been isolated from domestic pets,<sup>31, 32</sup> and meat products.<sup>33</sup> Multi locus sequence typing to determine genetic diversity of isolates from different mammalian species has not been performed, but would be interesting in terms of evaluating transmission sources. Similarly, studies that evaluate colonization in families living in the same house could provide information about transmission, as well as longitudinal studies investigating colonization in individuals.

### 5.1.4. Detection of virulence genes

In study III, we investigated the prevalence of two virulence associated genes that have previously been described as potentially pathogenic. The *zot* gene, encoding a toxin capable of destructing tight junctions, and exotoxin 9, associated to enhanced intracellular survival.<sup>149</sup> While we found no association to clinical presentation or sampling site for either gene, there was a genetic difference between isolates with exotoxin 9 and isolates with *zot*, with significant grouping into different GS. It was apparent that individuals could have isolates with different, or both virulence genes,

possibly explained by genetic exchange between isolates. We chose to examine these two putative virulence factors, since isolates with these genes have been associated to IBD and have shown to have pathogenic effects *in-vitro*.<sup>85, 149</sup> However, other genes, such as those encoding CRISPR-associated proteins,<sup>74</sup> or the sodium-hydrogen antiporter NhaC<sup>73</sup> have also been proposed to be associated to virulence in *C. concisus*, but these were not investigated in our present studies.

The genetic diversity of *C. concisus* has in part previously been explained by variations in genes encoding respiration pathways, peptidoglycan surface structures and flagellin glycosylation pathways.<sup>72, 73</sup> However, studies that examine genomic rearrangements or DNA exchange between strains are lacking. Evolution of bacterial pathogens within an individual is common, and has been described for other pathogens.<sup>156</sup> One of the factors that enables bacterial adaption in a host is phase variation (PV), the ability for bacteria to switch gene expression on and off by insertion or deletion of polyG or polyC repeat tracts located in the reading frames of genes during replication.<sup>157</sup> This has been described in detail for *C. jejuni*, in which most PV genes encode enzymes or surface proteins that can modify surface structures,<sup>157</sup> but this phenomenon has not been investigated in *C. concisus*. There is also very little description of horizontal gene transfer, or natural transformation in *C. concisus*. Natural transformation has been described for many bacterial species, and is also shown to occur independently of environmental influences in *C. jejuni*,<sup>158</sup> and is an important method for acquisition of resistance genes in bacteria. Chung and colleagues recently described the presence of a T4SS system, used to transport large molecules across cell membranes, in the genome of an enteric *C. concisus* strain. The system was not present in the oral strain from the same person, indicating that plasmid conjugation or gene transfer had occurred.<sup>74</sup>

## **5.2. Main conclusions**

We found that there was a high prevalence of viable *C. concisus* isolates in clinical samples from both IBD patients and healthy individuals. *Campylobacter concisus* was abundant in saliva, faecal samples as well as mucosal biopsies, indicating that colonization may be more extensive than previously assumed, and certainly is not restricted to the oral cavity. In our study, we included a unique group of IBD patients with previous UC-IPAA surgery. Patients with UC-IPAA have a history of severe UC, and had more clinical symptoms at the time of biopsy sampling than the other IBD groups in this study. When assessing the number of positive biopsies in total, there was a significantly higher prevalence of *C. concisus* in biopsies from UC-IPAA patients, compared to the other IBD groups and HC, which had similar prevalence rates. When assessing the number of patients that were positive in at least one biopsy, there was no difference between groups. This could indicate that it may be bacterial abundance, rather than bacterial presence, that contributes to inflammation.

By MLST analysis of *C. concisus* isolates from different patients and various locations, we confirmed previous findings of vast genetic diversity. While some patients were colonized with only one strain, others had several different strains, which did not correlate to clinical presentation. We showed that even isolates from the same gut mucosal biopsy could be genetically different, a finding previously only described for oral isolates.

MLST analysis did not reveal a correlation between certain *C. concisus* isolates and clinical presentation, indicating, similar to the findings in study I, that a possible association to disease could be related to relative quantities of *C. concisus*, instead of specific GS. Interestingly, there was an association to the site of sampling, with significantly more isolates from mucosal biopsies in GS II and oral isolates in GS I, however with no correlation to clinical presentation.

Our collection of Danish *C. concisus* isolates consisted mostly of gut mucosal isolates. We found a high prevalence of the putative virulence gene exotoxin 9, used as a proxy for the UNSWCD plasmid with four genes previously described only in isolates from patients with gastrointestinal disease. The prevalence of *zot* was considerably lower when comparing to previous findings from Australian oral isolates. Interestingly, the polymorphisms of the *zot* gene described in Australian isolates was not prevalent in our collection, possibly indicating variations between different sampling sites or maybe between the geographical origin of isolates. While we did not find any associations between virulence determinants and disease phenotype, these results should be interpreted with caution, since gene expression was not examined.

### **5.3. Clinical implications**

Isolation of single microbial organisms in relation to infectious diseases has become the fundament for understanding mechanisms of microbial pathogenic activity in relation to disease evolvement, since the introduction of Koch's postulates in 1890.<sup>159</sup> However as previously mentioned, the current understanding of chronic inflammatory bowel disease is that the etiology is multifactorial and complex, and that the gut microbiota as a whole plays an important part.<sup>17</sup> Whole genome sequencing technologies have provided a greater understanding of the human microbiome, and the modulations that can occur over time.<sup>5, 6, 13</sup> The microbiota of individuals with different *C. concisus* colonization status has not been investigated, but would be of great importance in recognizing the possible effects of other microbial species in the pathogenic capacity of *C. concisus*.

In a longitudinal study of five children with CD, Kaakoush et al. described an association between *C. concisus* and certain *Firmicutes* taxa (*Faecalibacterium* and *Lachnospiraceae* incertae sedis), since levels of these bacterial species in- and decreased symbiotically.<sup>76</sup> From the same clinical specimens, Kaakoush et al. described that abundance of the genera *Dialister* and *Oscillospira* were found to

correlate with higher levels of *zot* and Exotoxin 9 in CD children with *C. concisus* DNA in stool.<sup>81, 160</sup> Similarly to *C. concisus*, *Dialister invisus* is an oral bacterium that has been associated to periodontitis.<sup>34, 161</sup> Interestingly, in the description of the dysbiotic environment in CD, decreased levels of *Dialister invisus* have previously been associated to disease,<sup>162</sup> while *Oscillospira* spp. have been detected in increased levels in the submucosa of CD patients compared to controls.<sup>16</sup> The results from our studies indicate that a high abundance of *C. concisus* could be implicated in disease; it is conceivable that *C. concisus* has pathogenic capabilities that contribute to inflammation when the environmental circumstances allow it. Such circumstances could be deficient bacterial clearance, alterations in the epithelial mucus layer, or enriched availability of carbon sources through the metabolism of other microorganisms or metabolites from such organisms.<sup>163</sup> Studies that examine the effects of *C. concisus* in both symbiotic and dysbiotic gut microbiotas are warranted in order to further establish possible clinical implications of colonization.

Finally, the antibiotic susceptibility of *C. concisus* has been sparsely described. Vandenberg et al. tested 20 clinical *C. concisus* isolates from diarrheal stool samples (along with other non-*jejuni*/non-*coli* *Campylobacter* species) and found that 5% were resistant to erythromycin and ciprofloxacin,<sup>164</sup> which are antibiotic regimens commonly used in the treatment of diarrhea. In a large study from 441 clinical samples from patients with gastroenteritis from Aalborg, 16% of isolates were resistant to ciprofloxacin whereas macrolide resistance was very low.<sup>30</sup> In the five children with CD studied by Kaakoush et al, treatment with immune modulating drugs and antibiotics changed *C. concisus* colonization status, but the results were inconsistent and not related to inflammatory symptoms.<sup>76</sup> The results from our studies indicate that viable and potentially pathogenic *C. concisus* exist in the gastrointestinal tract of both healthy and diseased individuals. Therefore, the detection of *C. concisus* in a clinical sample should be interpreted with caution, and not necessarily indicate antibiotic treatment.



## CHAPTER 6. PERSPECTIVES

Although we found that *C. concisus* could be isolated from healthy individuals, previous demonstrations of the pathogenic potential of this bacteria should still sanction further investigations into possible associations to gastrointestinal disease. There are some accounts for possible associations to other gastrointestinal diseases such as microscopic colitis<sup>46</sup> – a subtype of IBD characterized by prolonged periods of diarrhea and inflammatory cell intrusion of the gut epithelium, without macroscopic evidence of inflammation.<sup>165</sup> The prevalence of *C. concisus* in esophageal conditions such as GERD, Barrett’s esophagus and EAC<sup>49</sup> also warrant further investigations into a possible association to these diseases. Recent, interesting findings in dysbiosis of the esophageal flora indicate that microbial dysbiosis may also be implicated in diseases of the upper gastrointestinal tract.<sup>166</sup>

Other molecular technologies are becoming increasingly available and automatized for routine use in clinical laboratories. While genomics provides us with detailed information about the genetic composition of bacteria, other “omics” should be included in order to truly comprehend the virulence of a pathogen. Transcriptomics is the measurement of mRNA in a cell, and can be described as a snapshot of gene expression. The most common method used for this is high throughput RNA sequencing (RNA-seq).<sup>83</sup> RNA-seq could elucidate whether or not a specific condition is required for expression of certain potential virulence genes, for example; if *zot* is expressed in the absence of inflammation. In proteomics, the quantity of expressed proteins are measured, and in metabolomics the metabolic state of an organism is evaluated by the total production of small molecules. All of these methods can provide valuable information about the pathogenic actions of bacteria and the dynamic interactions of bacteria in complex microbial communities.<sup>167</sup>

In conclusion, while our studies have provided insights into the abundance and genetic diversity of *C. concisus* in humans, there are many outstanding questions that still remain unanswered. Ongoing genetic investigation and experimental studies will hopefully expand our understanding of the enigmatic *C. concisus*, for which the terms commensal and pathogen both seem inadequate.

### **6.1. Additional publications and ongoing studies**

Kirk KF, Nielsen HL, Nielsen H. The susceptibility of *Campylobacter concisus* to the bactericidal effects of normal human serum, APMIS. 2015;123:269-74

Ovesen S., Kirk KF, Nielsen HL, Nielsen H. Motility of *Campylobacter concisus* isolated from saliva, faeces and gut mucosal biopsies, APMIS 2016. In press.

Nielsen HL, Kirk KF, Bodilsen J, Ejlersen T, Nielsen H. Azithromycin vs. placebo for the clinical outcome in *Campylobacter concisus* diarrhea in adults: A randomized, double-blinded, placebo-controlled clinical trial. PLoS One. 2016 Nov 28;11:e0166395

#### Ongoing studies:

Whole-genome characterization of the *C. concisus* core- and pan-genome

Microbiota profiling of *C. concisus* positive IBD patients and healthy controls

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# PAPERS

**Paper 1:** Optimized Cultivation of *Campylobacter concisus* from gut mucosal biopsies in inflammatory bowel disease

**Paper 2:** Comparing *Campylobacter concisus* strains from saliva, faeces and gut mucosal biopsies in inflammatory bowel disease by multi-locus sequence typing using whole genome sequence data

**Paper 3:** Virulence-associated genes in *Campylobacter concisus* isolates from patients with inflammatory bowel disease and healthy controls in Denmark



# APPENDICES

**Appendix A. Study participants**

**Appendix B. Protocol for cultivation of *C. concisus***

**Appendix C. Protocol for PCR**

**Appendix D. Overview of samples used for WGS**

**Appendix E. Assembled genome data and NCBI accession numbers**

**Appendix F. Phylogenetic trees**

# Appendix A. Study participants

ID	Group	Age	Sex	Surgery (year)	CRP (mg/L)	Leu (10 <sup>9</sup> /l)	Other illness / Medication	Notes	Clinical symptoms	Macroscopic*	Microscopic*	C.orientis Saliva (cul/PCR)	C.orientis Biopsies (cul/PCR)	C.orientis Faeces (cul/PCR)
1S	UC	60	M	-	1	4.0	5-ASA	-	Diarrhea 5-6/day	Inflammation in rectum	Chronic inactive inf.	0/1	0/1	0/0
2S	CD	30	M	-	1	8.2	ADA	-	Bloody stools	Rectal inf., edema	Chronic, inactive inf.	0/1	-	0/0
3S	UC	44	M	-	1.3	7.5	IFX, steroids	-	Bloody stools	Edema and inf. of the rectum	Chronic, active inf.	1/1	1/5	0/0
4S	CD	33	F	-	2	8.0	MCP	-	Increased stool freq.	Edema of the sigmoid & rectum	Chronic, active inf.	0/1	1/3	-
5S	CD	24	M	2014	13	7.0	ADA, steroids	-	Increased stool freq.	Severe edema at TI	Acute and chronic inflammation	1/1	0/4	0/0
6S	CD	43	M	1992 & 2011	16	8.7	MCP, IFX, steroids	1/3 SI resected	Increased stool freq.	Inf. at TI and rectum	Chronic, active inf.	1/1	0/0	0/0
7S	UC/IPAA	51	F	2007	7	8.1	-	-	Pouchitis	Ulcerations, frailty and fibrin	Chronic, active inf.	0/1	2/2	0/0
8S	UC/IPAA	41	M	1997	4	7.9	-	-	Pouchitis	Discreet infl.	Diffuse severe inflammation	1/1	1/1	0/0
9S	UC/IPAA	45	M	2007	24	7.2	-	Chronic AB refractory pouchitis	Pouchitis	Ulcerations and edema	Acute and chronic inflammation	1/1	3/3	0/1
10S	UC/IPAA	46	M	2007	1	8.5	-	-	Pouchitis	Ulcerations	Severe inflammation	1/1	1/0	1/1
11S	UC	63	F	-	3	6.0	5-ASA	-	Increased stool freq.	Inf. cecum to rectum	Chronic active inf. at cecum	1/1	2/6	0/0
12S	CD	63	F	-	8	10.8	IgA nephritis, MTX	-	Diffuse diarrhea	Normal	Normal	1/1	6/7	0/1
13S	UC/IPAA	55	F	1998	4	5.4	-	-	Pouchitis	Ulcerations	Diffuse inf.	1/0	1/2	0/0

14S	CD	34	F	2014	3	7.0	IFX, VDZ	Ileostomy, initial diag. was UC	Increased stoma output	-	-	1/1	-	1/1
15S	UC/IPAA	28	M	2011	1	6.2	-	-	Pouchitis	Normal	Nodular hyperplasia	0/1	3/2	0/0
16S	UC/IPAA	48	F	1999	3	5.0	-	Hodgkin's Lymphoma	Pouchitis	Ulcerations	Few ulcerations	0/1	1/2	1/1
17S	UC	35	F	2014	30	11.8	5-ASA	Ileostomy	Fecal incontinence	-	-	0/0	0/0	0/1
18S	UC/IPAA	31	M	2011	32	7.8	-	-	Pouchitis	Ulcerations	Chronic, active inf.	1/1	0/1	0/1
19S	CD	59	F	2014	5	14.0	-	Ileostomy	None	-	-	1/1	-	1/0
20S	UC/IPAA	56	F	1992	2	8.9	-	-	Pouchitis	Severe ulcerations	Chronic, active inf.	1/1	0/2	1/0
21S	UC/IPAA	26	F	2006	22	9.0	-	-	Pouchitis	Severe inflammation	Acute & chronic, inf.	0/0	0/1	-
22S	UC/IPAA	44	M	2008	14	6.3	-	Anal fistula	Pouchitis	Irritation w/o inflammation	Normal	0/1	2/2	1/1
23S	UC	69	F	-	0.9	6.4	-	-	Diarrhea	No inf.	Normal	1/1	0/2	0/1
24S	UC/IPAA	46	M	2011	1	5.9	-	-	Normal/ pouchitis	Normal	Unspec. reactive mucosa	0/0	0/2	0/1
26S	UC/IPAA	38	F	2011	4	8.5	-	Chronic AB refractory pouchitis	Pouchitis	Ulcerations and inf.	Chronic inf.	1/1	1/2	1/1
27S	UC/IPAA	49	F	1996	8	8.6	-	-	Pouchitis	Reactive cuff mucosa	Chronic inf. distal pouch	1/1	0/1	0/0
28S	CD	38	F	2005, 2013 & 2015	26	21.0	MTX, steroids	Ileocecal resection	Severe red. QOL	Inf. NT-ileum	Chronic, active inf.	1/1	0/0	-

29S	UC/IPAA	63	M	1988	2	9.5	-	-	-	Pouchitis	Ulcerations, mucosa frailty	Acute and chronic inf.	1/1	0/3	0/0
30S	CD	39	F	2000	5	15.4	MTX	Ileostomy & extraint. CD (joints)	Increased output	Increased output	-	-	1/1	-	1/0
21S	UC/IPAA	36	F	2014	0	4.9	-	-	-	Diarrhea	Normal	Normal	1/1	3/3	0/1
32S	UC/IPAA	58	M	2011	1	6.8	-	-	-	None	Normal	Normal	0/1	1/2	0/0
33S	CD	42	M	-	3	8.7	IFX, steroids	Kidney failure	None	None	Diffuse edema	Chronic inf.	1/1	0/0	0/0
34S	UC/IPAA	59	F	1993	13	10.1	-	-	-	Pouchitis	Inf. and ulcerations	Chronic inactive inflammation	1/1	0/2	0/1
35S	UC/IPAA	23	F	2014	-	-	-	-	-	Diarrhea	Normal	Chronic inactive inf	1/1	3/3	1/1
36S	UC	64	F	-	2-9	5.5	5-ASA	-	-	None	Normal	Normal	0/1	0/4	0/0
37S	UC/IPAA	49	M	2008	2	7.8	-	-	-	Pouchitis	Normal	Moderate inf.	1/1	0/3	1/0
38S	UC/IPAA	50	F	1995	2	10.9	ASA	-	-	Bloody stools	Diffuse inf.	Chronic inf.	1/1	0/2	0/0
39S	CD	30	M	-	13	8.2	ASA	-	-	Increased stool freq	Discreet inf.	-	1/1	3/4	1/1
40S	UC	51	M	-	1	3.7	ASA	-	-	Increased stool freq	Proctosigmoiditis	-	1/1	0/4	1/1
41S	CD	48	F	-	1	10.2	IFX, steroids	-	-	Abdominal pain	Normal	-	1/1	0/4	0/0
42S	UC	37	F	-	0	7.2	5-ASA	-	-	Increased stool freq	Inf. rectum	Acute and chronic inflammation	1/1	0/1	0/0

43S	UC	67	F	-	6	12	5-ASA	-	None	Normal	Normal	1/1	3/5	1/0
44S	UC	36	M	-	3	10.2	-	PSC	None	Diffuse inf.	Chronic, inactive inf.	1/1	2/6	0/0
45S	UC	61	F	-	-	-	5-ASA, steroids	No COLS	Bloody and slimy stools	-	-	-	-	0/0
46S	UC	59	M	-	1.9	4.8	-	-	None	Normal	Normal	1/1	0/3	0/0
47S	UC/IPAA	38	F	2007	4	7.7	-	-	None	Normal	Prox. chronic inactive inf.	1/1	1/3	0/1
48S	UC/IPAA	21	M	2010	1.5	6.1	-	-	Increased stool freq.	Normal	Reactive mucosa	1/1	2/3	0/1
49S	UC/IPAA	38	F	2001	34	9.0	-	-	Pouchitis	Severe ulcerations	Acute and chronic inf.	1/1	3/3	1/1
50S	UC	50	F	-	-	-	-	-	None	Diffuse rednes	Chronic, inactive inf.	1/1	0/0	0/0
51S	UC/IPAA	41	M	2012	0.5	5.0	-	-	Pouchitis	Normal	Chronic, inactive inf.	1/1	0/1	1/1
52S	UC	59	M	-	0.8	5.5	5-ASA, steroids, VDZ	-	None	Normal	-	1/1	2/3	0/1
54S	UC	33	M	-	9.3	7.4	ADA, steroids	Inf. in SI	Increased stool freq. & abdominal pain	Inf. terminal ileum (capsule endo: inf. SI)	-	0/0	0/0	0/0
55S	CD	57	F	2005	1.8	7.4	Steroids	-	Abdominal pain	Inf. anastomis	Reactive inf.	1/1	1/2	0/0
56S	UC	36	M	-	1.5	6.0	5-ASA	PSC	None	Normal mucosa	-	0/0	0/0	0/0
58S	UC	68	F	-	-	6.3	5-ASA	-	None	Normal mucosa	-	1/1	0/1	-

59S	UC/PAA	42	M	2000	3.4	6.4	-	-	None	Normal pouch, one ulceration	Normal pouch, active inf. ulcer	1/1	2/3	0/0
60S	UC/PAA	60	M	2003	0.5	4.0	-	PSC	Pouchitis	Pouchitis	Chronic inf.	1/1	0/0	1/1
1D	D	57	F	-	1.0	9.0	-	-	Diarrhea > 12 weeks	Normal	Normal	0/1	4/5	0/0
2D	D	20	F	-	0.5	5.7	-	Recent travels (Colaba)	Diarrhea > 4 weeks	Normal	Normal	1/1	0/0	1/1
2R	HC	73	M	-	-	-	-	Fam. disp. to CRC***	None	Normal	-	1/1	2/6	0/0
3R	HC	66	M	-	-	-	-	HNPCC, MM	None	Normal	-	1/1	2/4	0/1
4R	HC	27	M	-	-	-	-	HNPCC	None	Normal	-	1/1	0/0	0/0
5R	HC	68	M	-	1.8	6.3	-	HNPCC	None	Diverticulosis	-	0/0	0/0	0/0
6R	HC	66	M	-	0.6	5.9	-	HNPCC	None	Normal	-	0/1	1/5	0/0
7R	HC	51	M	-	-	-	-	No COLS, insuf. prep	None	-	-	1/1	-	0/0
8R	HC	45	F	-	-	-	-	Fam. disp. to CRC***	None	Normal	-	1/1	0/0	0/0
9R	HC	57	M	-	-	-	-	Adenoma	None	Polypsosis	-	1/1	2/3	0/1
10R	HC	63	F	-	0.9	6.8	-	Prev. DVI	None	Diverticulosis	-	0/1	2/3	0/0
11R	HC	66	F	-	0	6.9	-	HNPCC	None	Normal	-	1/1	0/6	1/1



12R	HC	56	M	-	0	4.2	-	HNpCC	None	Normal	-	1/1	0/5	1/0
13R	HC	49	F	-	5	9.1	-	HNpCC	None	Normal	-	1/1	0/4	0/0
14R	HC	69	M	-	1	5.1	-	Fam. disp. to CRC***	None	Normal	-	0/0	1/4	0/0
15R	HC	53	M	-	-	-	-	Fam. disp. to CRC***	None	Normal	-	0/1	1/5	0/0
16R	HC	47	F	-	-	-	-	Fam. disp. to CRC***	None	Normal	-	1/1	0/3	0/0
17R	HC	40	F	-	0.5	7.3	-	Fam. disp. to CRC***	None	Polyps	No dysplasia	0/1	0/3	0/0
18R	HC	65	F	-	4	5.8	-	HNpCC	None	Normal	-	0/1	0/3	0/1
19R	HC	67	F	-	4	4.6	MTX (RA)	Prev. DVI	None	Diverticulosis	-	0/0	0/0	1/0
20R	HC	49	F	-	-	-	Breastcancer	Fam. disp. to CRC***	None	Normal	-	1/1	4/5	0/0
22R	HC	51	M	-	-	-	-	HNpCC	None	Normal	-	1/1	0/0	0/0
23R	HC	71	F	-	21	6.5	-	Prev. DVI	None	Diverticulosis	-	1/1	0/0	0/0
24R	HC	57	M	-	-	-	-	Fam. disp. to CRC***	None	Normal	-	1/1	7/6	0/1
25R	HC	30	M	-	-	-	-	HNpCC	None	Normal	-	1/1	0/1	-
26R	HC	52	F	-	-	-	-	HNpCC	None	Normal	-	1/1	0/0	0/0

27R	HC	51	F	-	-	-	-	-	Prev. DVI	None	Diverticulosis	-	1/1	0/0	0/0
28R	HC	57	F	-	-	-	-	-	HNPCC	None	Polyps	-	1/1	0/0	0/0

\* Macroscopic findings as reported by the physician at the time of performing the colonoscopy when biopsies were taken.

\*\* Microscopic findings as reported by the pathologist in cases where biopsies were sent for histological examination (off protocol).

\*\*\* Family history of colorectal cancer, other than HNPCC.

Abbreviations: AB: antibiotics, ADA: Adalimumab (Humira), 5-ASA: Mesalazin, CRC: Colon cancer, COLS: colonoscopy, inf: inflammation, DVI: Diverticulitis, HNPCC: Hereditary, non-polyposis colorectal cancer, IFX: Infliximab (Remicade) LI: Large intestine, MCP: Mercaptopurine (Purinethol, Imurel), MM: Myelomatosis, MTX: Methotrexate, NT-ileum: neo-terminal ileum, PSC: Primary sclerosing cholangitis, QOL: Quality of life, RA: Rheumatoid arthritis, SI: small intestine, TI: terminal ileum, VDZ: vedolizumab, wks: weeks

# Appendix B. Protocol for cultivation of *C. concisus* from different locations

## Materials:

Agarplates: 5% blood agar plates with added yeast extract  
Polycarbonate filters (47 mm diameter, 6 µm pore size)  
10µl inoculation loops  
2 ml Eppendorf tubes  
Sterile saline  
Cotton tip swabs

## Incubation conditions:

Microaerobic (MA): 80% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% H<sub>2</sub>, 6% O<sub>2</sub>  
Anaerobic (A): 80% N<sub>2</sub>, 10% CO<sub>2</sub>, 10% H<sub>2</sub>

## Protocol:

- 1) Collection of samples (transport to lab immediately after collection):
  - a. Biopsies are collected with sterile forceps and placed in sterile containers with 0.5 ml sterile saline.
  - b. Stool samples are collected in dry containers by participants and sent by mail to the laboratory.
  - c. Saliva samples are collected in dry, sterile containers on the day of colonoscopy.
- 2) Protocol for faecal samples:
  - a. Homogenize faecal sample by mixing with sterile saline 1:1.
  - b. Mount 4-5 drops of emulsion to polycarbonate filter on 5% blood agar with yeast extract.
  - c. Incubate for 1 hour at room temperature
  - d. Remove filter with sterile forceps.
  - e. Incubate for 96 hours in MA atmosphere.
  - f. Inspect plates daily – colonies that resemble *Campylobacter* spp. Are inspected by
    - i. Wet-mount microscopy
    - ii. MALDI-TOF analysis
    - iii. qPCR analysis (primers Conc Fmod and Cons R2)
- 3) Protocol for saliva samples:
  - a. Collect saliva on a cotton swab and streak onto agarplate, covering the entire surface.
  - b. Place 2 x Vancomycin tablets (5 mg) on the agar plate.
  - c. Incubate in MA atmosphere for 2 days.

- d. Collect bacterial mass from 5 mm zone around vancomycin tablets and liquefy in Eppendorf tube with 50  $\mu$ l sterile saline.
  - e. Follow steps 2b-2f.
- 4) Protocol for biopsies:
- a. Biopsies are smeared onto 2 agar plates using inoculation loops.
  - b. One plate is incubated for 48 hours in MA, the other in A atmospheres.
  - c. Following 48 hours of incubation: harvest approximately 100  $\mu$ g bacterial mass by streaking across the agar plates with inoculation loop.
  - d. Place bacterial mass in Eppendorf tube and add 50  $\mu$ l sterile saline – vortex briefly.
  - e. Follow steps 2b-2f.

# Appendix C. Protocol for PCR for detection of *C. concisus* DNA in mucosal biopsies

- 1) Preparation of DNA from gut mucosal biopsies:
  - a. Place biopsy in 1.5 mL Eppendorf tube and add 500  $\mu$ L sterile saline and 1 stainless steel bead (Qiagen)
  - b. Homogenize in Tissue Lyser for 2 min. at 20 Hz according to instructions
- 2) DNA extraction (EasyMaG Nuclisens, BioMeriueux)
  - a. Extraction as per on-board protocol A to final eluate of 110  $\mu$ L.
  - b. DNA is stored at -20 °C until used for PCR analysis.
- 3) Preparation for bacterial 16S rRNA gene PCR:
  - a. Materials: SYBR green Mastermix (x2), F and R primers, template DNA, sterile saline in the following mix in a 96 well PCR plate:

Reagent	Volume
SYBR Green Mastermix (x2)	12.5 $\mu$ L
Forward primer (F27)	1 $\mu$ L
Reverse Primer (R1494)	1 $\mu$ L
Template DNA	2 $\mu$ L
Sterile H2O	8.5 $\mu$ L
<b>Total</b>	<b>25 <math>\mu</math>L</b>

Primer conc.: 10 $\mu$ M x 1  $\mu$ L=25 $\mu$ L x 0.4  $\mu$ M=400 nM

Remember to account for positive and negative controls and “waste”.

- 4) Centrifuge 96 well plate for 1 min at 3000 rpm before PCR.
- 5) PCR is conducted using real-time qPCR (7500 Applied Biosystems) with the following conditions:

Primer	Start	Dissociation	Annealing	Elongation	Cycles
<b>16S rRNA (bacterial)</b>	95°C, 10 min	95°C, 20 sec.	63°C, 20 sec.	72°C, 1 min.	35
<i>C. concisus</i>	95°C, 10 min	95°C, 30 sec.	60°C, 30 sec.	72°C, 30 sec.	40

- 6) Verification of product size:
  - a. Run 96 well plate on QiaExcel Advanced Screen Gel:

- i. Add 15 µl dilution buffer to empty wells.
- ii. Add 10 µl size marker (mastermix: 25µl SYBR green, 35µl sterile saline and 30 µl size marker)
- iii. Add alignment markers (15µl) to single cartridge
- iv. Run using profile “DEC”

7) PCR product purification:

- a. Use Qiagen purification kit according to instructions.

8) PCR for *C. concisus* 16S rRNA gene

- a. Materials: SYBR green Mastermix (x2), F and R primers, template DNA, sterile saline in the following mix in a 96 well PCR plate:

Reagent	Volume
SYBR Green Mastermix (x2)	12.5 µL
Forward primer (ConcFmod)	1.25 µL
Reverse Primer (Conc R2)	1.25 µL
Template DNA	5 µL
Sterile H2O	5 µL
<b>Total</b>	25 µL

Primer conc.:  $10\mu\text{M} \times 1\mu\text{L} = 25\mu\text{L} \times 0.4\mu\text{M} = 400\text{ nM}$

Remember to account for positive and negative controls and “waste”

9) Repeat steps 4 – 6 for PCR and product size verification.

# Appendix D. Isolates used for WGS

ID	Group	Age	Sex	Isolate ID	Source	Location (of biopsy)
3UC	UC	44	M	AAUH-3UCce	Biopsy	Cecum
				AAUH-3UCce2	Biopsy	Cecum 2
4UC	UC	33	F	AAUH-4UCti	Biopsy	Terminal ileum
				AAUH-4UCti-a	Biopsy	Terminal ileum
5CD	CD	24	M	AAUH-5CDo	Saliva	
7UC	UC-IPAA	51	F	AAUH-7UCil	Biopsy	Ileum
8UC	UC-IPAA	41	M	AAUH-8UCo	Saliva	
				AAUH-8UCpp	Biopsy	Proximal pouch
				AAUH-8UCpp-a	Biopsy	Proximal pouch
9UC	UC-IPAA	45	M	AAUH-9UCpp	Biopsy	Proximal pouch
				AAUH-9UCdp	Biopsy	Distal pouch
10UC	UC-IPAA	46	M	AAUH-10UCf2	Faeces	
				AAUH-10UCil-a	Biopsy	Ileum
11UC	UC	63	F	AAUH-11UCco	Saliva	
				AAUH-11UCdes-a	Biopsy	Descending colon
				AAUH-11UCsig-a	Biopsy	Sigmoideum
12CD	CD	63	F	AAUH-12CDo	Saliva	
				AAUH-12CDti2-a	Biopsy	Terminal ileum 2
				AAUH-12CDti4-a	Biopsy	Terminal ileum 4
				AAUH-12CDti5-a	Biopsy	Terminal ileum 5
				AAUH-12CDce	Biopsy	Cecum
				AAUH-12CDtra-a	Biopsy	Transverse colon
				AAUH-12CDtra2-a	Biopsy	Transverse colon 2
				AAUH-12CDdes2	Biopsy	Descending colon 2
				AAUH-12CDdes3	Biopsy	Descending colon 3
				AAUH-12CDdes4	Biopsy	Descending colon 4
				AAUH-12CDsig	Biopsy	Sigmoideum
				AAUH-12CDrec-a	Biopsy	Rectum
15UC	UC-IPAA	28	M	AAUH-15UCpp	Biopsy	Proximal pouch
				AAUH-15UCdp	Biopsy	Distal pouch
				AAUH-15UCdp-a	Biopsy	Distal pouch
16UC	UC-IPAA	48	F	AAUH-16UCo-a	Saliva	
				AAUH-16UCdp	Biopsy	Distal pouch
				AAUH-16UCdp3	Biopsy	Distal pouch 3
				AAUH-16UCdp5	Biopsy	Distal pouch 5
				AAUH-16UCf	Faeces	
				AAUH-16UCf2	Faeces	
				AAUH-16UCf3	Faeces	
20UC	UC-IPAA	56	F	AAUH-20UCco	Saliva	
				AAUH-20UCf	Faeces	
22UC	UC-IPAA	44	M	AAUH-22UCpp-a	Biopsy	Proximal pouch
35UC	UC-IPAA	23	F	AAUH-35UCil-a	Biopsy	Ileum
				AAUH-35UCil2-a	Biopsy	Ileum 2
				AAUH-35UCil3-a	Biopsy	Ileum 3
				AAUH-35UCil4-a	Biopsy	Ileum 4
				AAUH-35UCpp	Biopsy	Proximal pouch
				AAUH-35UCdp	Biopsy	Distal pouch
37UC	UC-IPAA	49	M	AAUH-35UCf	Faeces	
				AAUH-37UCo-a	Saliva	
39CD	CD	30	M	AAUH-37UCf	Faeces	
				AAUH-39CDti-a	Biopsy	Terminal ileum
				AAUH-39CDrec-a	Biopsy	Rectum
40UC	UC	51	M	AAUH-39CDF	Faeces	
				AAUH-40UCf	Faeces	
43UC	UC	67	F	AAUH-43UCce-a	Biopsy	Cecum
				AAUH-43UCf	Faeces	
44UC	UC	36	M	AAUH-44UCsig-a	Biopsy	Sigmoideum
				AAUH-44UCsig6	Biopsy	Sigmoideum 6
47UC	UC-IPAA	38	F	AAUH-47UCil	Biopsy	Ileum
				AAUH-47UCil-a	Biopsy	Ileum
48UC	UC-IPAA	21	M	AAUH-48UCo-a	Saliva	
				AAUH-48UCil-a	Biopsy	Ileum
				AAUH-48UCdp-a	Biopsy	Distal pouch
49UC	UC-IPAA	38	F	AAUH-49UCil-a	Biopsy	Ileum
				AAUH-49UCpp-a	Biopsy	Proximal pouch
				AAUH-49UCf	Faeces	Faeces

51UC	UC-IPAA	41	M	AAUH-51UCf	Faeces	
55UC	UC	57	F	AAUH-55UCtra-a	Biopsy	Transverse colon
58UC	UC	68	F	AAUH-58UCo	Saliva	
59UC	UC-IPAA	42	M	AAUH-59UCpp-a	Biopsy	Proximal pouch
2HC	HC	73	M	AAUH-2HCtra	Biopsy	Transverse colon
3HC	HC	44	M	AAUH-3HCco	Saliva	
				AAUH-3HCce2	Biopsy	Cecum 2
6HC	HC	66	M	AAUH-6HCco-a	Saliva	
8HC	HC	45	F	AAUH-8HCco	Saliva	
				AAUH-8HCco-a	Saliva	
9HC	HC	57	M	AAUH-9HCce	Biopsy	Cecum
				AAUH-9HCasc	Biopsy	Ascending colon
10HC	HC	63	F	AAUH-10HCce	Biopsy	Cecum
				AAUH-10HCdes	Biopsy	Descending colon
				AAUH-10HCdes2	Biopsy	Descending colon 2
				AAUH-10HCdes3	Biopsy	Descending colon 3
				AAUH-10HCdes4	Biopsy	Descending colon 4
				AAUH-10HCdes5	Biopsy	Descending colon 5
				AAUH-10HCdes6	Biopsy	Descending colon 6
				AAUH-10HCdes7	Biopsy	Descending colon 7
				AAUH-10HCtra	Biopsy	Transverse colon
11HC	HC	66	F	AAUH-11HCf	Faeces	
				AAUH-11HCco-a	Saliva	
12HC	HC	56	M	AAUH-12HCf	Faeces	
14HC	HC	69	M	AAUH-14HCce	Biopsy	Cecum
15HC	HC	53	M	AAUH-15HCti	Biopsy	Terminal ileum
19HC	HC	67	F	AAUH-19HCf	Faeces	
				AAUH-19HCf2	Faeces	
20HC	HC	49	F	AAUH-20HCasc	Biopsy	
				AAUH-20HCsig-a	Biopsy	Sigmoideum
				AAUH-20HCrec-a	Biopsy	Rectum
1D	D	57	F	AAUH-1Dce	Biopsy	Cecum
				AAUH-1Dasc	Biopsy	Ascending colon
				AAUH-1Dtra	Biopsy	Transverse colon
				AAUH-2012179281	Faeces	*
20103762 21	D	65	M	AAUH-2010376221	Faeces	
2D	D	20	F	AAUH-25Df	Faeces	
				AAUH-25Df3	Faeces	**
Ref.	HC			ATCC 51561	Faeces	
Ref.	CD	12	M	UNSW3	Biopsy	
Ref.	D			UNSW1	Faeces	
Ref.	CD			UNSWCD	Biopsy	
Ref.	D			UNSWCS	Faeces	
Ref.	D			ATCC 51562	Faeces	
Ref.	CD	3	M	UNSW2	Biopsy	
Ref.	D			13826	Faeces	
Ref.	Gingivitis			ATCC 33237	Saliva	

Abbreviations: CD: Crohn's disease, UC: Ulcerative colitis, UC-IPAA: J-pouch, HC: healthy control, D: Diarrhea. Isolate ID's indicate patient and isolate source. Isolates ending in -a indicate derivation from cultivation in anaerobic atmosphere, all others derive from microaerobic incubation. Ref.: reference isolates from ncbi.gov. Individual isolates from the same location (i.e. same agar plate) are numerated accordingly. Isolates with only numerical IDs derive from previous studies on patients with persistent diarrhea.\* Isolate from faeces taken 4 years prior to isolates from the current study \*\*Isolate derived from different faecal sample taken three months after initial faecal sample and after two weeks treatment with ciprofloxacin.



# Appendix E. Assembled genome data and BIGSdb IDs

	Isolate ID	Disease	Source	Genome size (Mbp)	Contigs	N50	GC (%)	BIGSdb ID
1	AAUH-3UCce	UC	Biopsy	1.93	44	103045	39.49	5640
2	AAUH-3UCce2	UC	Biopsy	1.93	38	101107	39.5	5645
3	AAUH-4UCti	UC	Biopsy	1.98	54	97028	39.26	5633
4	AAUH-4UCti-a	UC	Biopsy	1.98	51	97971	39.2	5632
5	AAUH-5CDo	CD	Saliva	2.10	183	89584	37.26	5616
6	AAUH-7UCil	UC-IPAA	Biopsy	1.98	58	95686	39.44	5634
7	AAUH-8UCo	UC-IPAA	Saliva	1.99	41	154956	39.48	5631
8	AAUH-8UCpp	UC-IPAA	Biopsy	2.02	3	82883	39.46	5625
9	AAUH-8UCpp-a	UC-IPAA	Biopsy	1.87	92	39071	39.66	5657
10	AAUH-9UCpp	UC-IPAA	Biopsy	1.82	47	79679	39.64	5625
11	AAUH-9UCdp	UC-IPAA	Biopsy	2.00	102	59846	39.46	5662
12	AAUH-10UCf2	UC-IPAA	Faeces	1.99	36	180736	39.59	5618
13	AAUH-10UCil-a	UC-IPAA	Biopsy	2.08	103	180889	39.41	5599
14	AAUH-11UCo	UC	Saliva	1.89	112	161715	37.46	5651
15	AAUH-11UCdes-a	UC	Biopsy	1.91	51	58626	37.3	5650
16	AAUH-11UCsig-a	UC	Biopsy	1.99	31	159029	39.48	5629
17	AAUH-12CDo	CD	Saliva	1.81	29	197548	37.56	5670
18	AAUH-12CDti2-a	CD	Biopsy	1.81	9	934037	37.56	5612
19	AAUH-12CDti4-a	CD	Biopsy	1.81	34	83693	37.57	5669
20	AAUH-12CDti5-a	CD	Biopsy	1.82	29	187882	37.59	5665
21	AAUH-12CDce	CD	Biopsy	1.86	31	483873	37.46	5659
22	AAUH-12CDtra-a	CD	Biopsy	1.97	58	63299	39.42	5636
23	AAUH-12CDtra2-a	CD	Biopsy	1.81	24	266692	37.59	5664
24	AAUH-12CDdes2	CD	Biopsy	1.85	49	158794	37.53	5661
25	AAUH-12CDdes3	CD	Biopsy	1.81	54	77176	37.64	5613
26	AAUH-12CDdes4	CD	Biopsy	1.85	26	158715	37.46	5660
27	AAUH-12CDsig	CD	Biopsy	1.90	20	257808	37.34	5655
28	AAUH-12CDrec-a	CD	Biopsy	1.91	52	102065	39.63	5649
29	AAUH-15UCpp	UC-IPAA	Biopsy	1.90	36	162832	39.66	5652
30	AAUH-15UCdp	UC-IPAA	Biopsy	2.06	57	52907	39.47	5621
31	AAUH-15UCdp-a	UC-IPAA	Biopsy	2.03	130	35098	39.51	5617
32	AAUH-16UCo-a	UC-IPAA	Saliva	1.88	43	151248	37.68	5656
33	AAUH-16UCdp	UC-IPAA	Biopsy	1.92	57	162832	39.69	5646
34	AAUH-16UCdp3	UC-IPAA	Biopsy	1.99	66	64884	39.67	5630
35	AAUH-16UCdp5	UC-IPAA	Biopsy	2.04	39	142542	39.48	5620
36	AAUH-16UCf	UC-IPAA	Faeces	1.89	33	392237	37.68	5654
37	AAUH-16UCf2	UC-IPAA	Faeces	1.88	61	64374	37.67	5610
38	AAUH-16UCf3	UC-IPAA	Faeces	1.87	138	21709	37.69	5658
39	AAUH-20UCo	UC-IPAA	Saliva	1.81	24	235464	37.58	5667
40	AAUH-20UCf	UC-IPAA	Faeces	1.91	81	45189	39.7	5609
41	AAUH-22UCpp-a	UC-IPAA	Biopsy	2.02	44	131008	39.58	5598
42	AAUH-35UCil-a	UC-IPAA	Biopsy	1.93	55	97085	39.54	5642
43	AAUH-35UCil2-a	UC-IPAA	Biopsy	1.93	41	138370	39.52	5647
44	AAUH-35UCil3-a	UC-IPAA	Biopsy	1.93	39	97130	39.52	5648
45	AAUH-35UCil4-a	UC-IPAA	Biopsy	1.94	61	80285	39.49	5641
46	AAUH-35UCpp	UC-IPAA	Biopsy	1.93	40	134251	39.47	5644
47	AAUH-35UCdp	UC-IPAA	Biopsy	1.96	70	138943	39.55	5635
48	AAUH-35UCf	UC-IPAA	Faeces	1.97	76	87016	39.52	5637
49	AAUH-37UCo-a	UC-IPAA	Saliva	1.90	41	89957	37.49	5653
50	AAUH-37UCf	UC-IPAA	Faeces	2.00	54	87543	39.52	5623
51	AAUH-39CDti-a	CD	Biopsy	1.93	62	120353	39.47	5643
52	AAUH-39CDrec-a	UCD	Biopsy	2.01	94	42323	39.38	5626
53	AAUH-39CDF	CD	Faeces	1.96	54	136855	39.51	5639
54	AAUH-40UCf	UC	Faeces	2.11	178	34887	39.42	5614
55	AAUH-43UCce-a	UC	Biopsy	2.01	118	73289	39.76	5619
56	AAUH-43UCf	UC	Faeces	1.97	67	62023	39.64	5638
57	AAUH-44UCsig-a	UC	Biopsy	2.05	111	54029	39.40	5708
58	AAUH-44UCsig6	UC	Biopsy	2.02	235	16737	39.56	5706
59	AAUH-47UCil	UC-IPAA	Biopsy	1.94	69	70435	39.78	5697
60	AAUH-47UCil-a	UC-IPAA	Biopsy	1.92	90	42174	39.81	5690
61	AAUH-48UCo-a	UC-IPAA	Saliva	1.88	80	45066	37.58	5679
62	AAUH-48UCil-a	UC-IPAA	Biopsy	1.97	58	65623	39.70	5699

63	AAUH-48UCdp-a	UC-IPAA	Biopsy	1.94	202	19218	39.78	5695
64	AAUH-49UCil-a	UC-IPAA	Biopsy	2.12	160	43381	39.27	5713
65	AAUH-49UCpp-a	UC-IPAA	Biopsy	2.1	162	52807	39.59	5712
66	AAUH-49UCf	UC-IPAA	Faeces	2.22	118	97148	39.15	5715
67	AAUH-51UCf	UC-IPAA	Faeces	1.9	61	100202	37.29	5684
68	AAUH-55UCtra-a	UC	Biopsy	1.88	88	37875	37.43	5677
69	AAUH-58UCo	UC	Saliva	2.02	105	34563	39.47	5705
70	AAUH-59UCpp-a	UC-IPAA	Biopsy	1.92	49	102305	39.62	5691
71	AAUH-2HCtra	HC	Biopsy	1.99	84	52482	39.42	5703
72	AAUH-3HC	HC	Saliva	2.03	336	26870	37.93	5711
73	AAUH-3HCce2	HC	Biopsy	2.07	328	33756	39.88	5714
74	AAUH-6HCco-a	HC	Saliva	1.78	85	32468	37.59	5671
75	AAUH-8HC	HC	Saliva	1.78	146	23267	37.76	5672
76	AAUH-8HCco-a	HC	Saliva	1.81	132	25221	37.64	5674
77	AAUH-9HCce	HC	Biopsy	1.98	189	21463	39.71	5701
78	AAUH-9HCasc	HC	Biopsy	1.80	86	41150	37.68	5673
79	AAUH-10HCce	HC	Biopsy	1.94	84	43541	39.61	5693
80	AAUH-10HCdes	HC	Biopsy	1.91	55	65110	39.58	5687
81	AAUH-10HCdes2	HC	Biopsy	1.90	79	59463	39.62	5681
82	AAUH-10HCdes3	HC	Biopsy	1.91	59	74769	39.59	5686
83	AAUH-10HCdes4	HC	Biopsy	1.90	90	45360	39.64	5680
84	AAUH-10HCdes5	HC	Biopsy	1.91	84	39333	39.6	5685
85	AAUH-10HCdes6	HC	Biopsy	1.98	102	36601	39.63	5700
86	AAUH-10HCdes7	HC	Biopsy	1.90	74	50724	39.62	5682
87	AAUH-10HCtra	HC	Biopsy	1.95	72	60348	39.59	5696
88	AAUH-11HCf	HC	Faeces	1.92	229	14154	37.71	5688
89	AAUH-11HCco-a	HC	Saliva	1.93	218	14905	37.7	5692
90	AAUH-12HCf	HC	Faeces	1.96	161	22427	39.49	5698
91	AAUH-14HCce	HC	Biopsy	1.83	157	21790	37.66	5675
92	AAUH-15HCti	HC	Biopsy	1.95	129	24790	39.84	5694
93	AAUH-19HCf	HC	Faeces	2.01	85	48338	39.51	5704
94	AAUH-19HCf2	HC	Faeces	1.98	155	22062	39.64	5702
95	AAUH-20HCasc	HC	Biopsy	2.04	319	20031	39.81	5710
96	AAUH-20HCsig-a	HC	Biopsy	2.04	152	33006	39.43	5709
97	AAUH-20HCrec-a	HC	Biopsy	2.00	82	84768	39.31	5707
98	AAUH-1Dce-a	D	Biopsy	2.2	356	48282	39.56	5716
99	AAUH-1Dasc	D	Biopsy	1.88	234	13858	39.77	5678
100	AAUH-1Dtra	D	Biopsy	1.91	93	39458	39.56	5683
101	AAUH-2012179281	D	Faeces	1.92	34	138913	37.35	5689
102	AAUH-2010376221	D	Faeces	1.85	84	48012	37.67	5676
103	AAUH-25Df	D	Faeces	1.81	31	134513	39.74	5666
104	AAUH-25Df3	D	Faeces	2.01	51	121167	39.38	5624
	ATCC 51561 (ref)							5357*
	UNSW3 (ref)							5358*
	UNSW1 (ref)							5359*
	UNSWCD (ref)							5360*
	UNSWCS (ref)							5361*
	ATCC 51562 (ref)							5362*
	UNSW2 (ref)							5363*
	13826 (BAA-1457) (ref)							5369*
	ATCC 33237 (ref)							5398*

\* Corresponding NCBI accession numbers: ATCC 51561= ANNH000000000, UNSW3= ANNE000000000, UNSW1= ANNf000000000, UNSWCD= AENQ000000000, UNSWCS= ANNG000000000, ATCC 51562= ANNI000000000, UNSW2= ANNJ000000000, 13826=CP000792.1, CP000793.1, CP000794.1, ATCC 33237= NZ\_CP012541.1



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